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PITUITARY-DIABETES IN THE CAT; RECOVERY FOLLOWING INSULIN OR DIETARY TREATMENT

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FOLLOWING THE ANNOUNCEMENT (1) that permanent diabetes could be produced in dogs by means of anterior pituitary extract, Young (2) reported experiments in other species. He administered crude anterior pituitary extract to mice, rats, guinea pigs, rabbits and cats. Of these animals, the mouse, rat and guinea pig were "almost completely insensitive" to the diabetogenic action of the extract. Rabbits and cats showed slight and transitory glycosuria in 50 per cent or less of the animals tested, whereas all but 1 of 25 dogs developed glycosuria.

In the present work the production of pituitary-diabetes in cats was undertaken, *a*), to learn whether the native resistance to pituitary extract could be overcome by partial pancreatectomy in this species as it had been in the rat (3) and if so, whether permanent diabetes could be produced; and, *b*), to develop a smaller and more economical animal than the dog for the study of this condition. These questions have been answered by the successful production of permanent diabetes in the partially depancreatized cat. *c*). In addition the cat has displayed certain differences from the dog (4) in the course and nature of the disease, as a result of which, *d*), it has been possible to study the reversibility (5) of early diabetes. Insulin treatment and reduction in diet have led to the recovery of these animals under the conditions described herein. The literature of pituitary-diabetes has been reviewed by Marks and Young (6) and the articles concerning the prevention or recovery of experimental diabetes will be reviewed with the results.

METHODS

Animals. Normal cats of both sexes were observed for several days and those which were free of respiratory infection and eating well were selected for the experiments. In order to produce permanent diabetes in the cat it was necessary to remove part of the pancreas prior to the injection of anterior pituitary extract. In all operations, pentobarbital sodium (nembutal) has been the anesthetic. This has been given intraperitoneally in doses of 35

to 45 mg. per kilogram. All operations have been performed under aseptic technique. At partial pancreatectomy from ¼ to ½ of the pancreas was left in place without damaging the pancreatic or bile ducts. The entire splenic portion and that overlying the portal vein have always been removed.

After operation the animals were kept in metabolism cages, fed measured diets, and the urine was tested daily for glucose and acetone bodies until the animals were autopsied. After 3 weeks the animals which ate well and were in good condition were ready for the injection of anterior pituitary extract. This period of observation on an ample diet served to identify the few animals which became spontaneously diabetic as a result of the operation. In some of the animals, blood sugar estimations and glucose tolerance tests were also performed; the latter too infrequently to be included in the results.

Extract. Crude saline anterior pituitary extract (APE) was prepared by Young's (2) method from carefully frozen and dissected beef glands.[1] We have described this preparation either as cc. of extract or as grams of gland used in making the extract (2 cc. extract = 1 gm. of gland). Such extracts do not represent the total extractable material, as noted by Young (2). This was injected intraperitoneally in doses of 2 to 10 cc. daily until persistent diabetes was established, or until the animal failed to respond. As a rule, 5 cc. per day was adequate and doses above 10 cc. have rarely seemed helpful. Unlike the normal dog, the partially depancreatized cat usually did not require increasing amounts of extract to maintain its glycosuria, although small increments were sometimes made. The observation of persistent diabetes for long periods (1 to 9 months) has led us to consider that the presence of glycosuria at the end of 2 weeks or a marked glycosuria 6 days after stopping pituitary extract indicated permanent diabetes. In animals *not* made permanently diabetic, the glycosuria disappeared in 1 to 6 days after extract was discontinued as noted by Houssay (7) in the dog.

At the time of writing, more than 25 permanently diabetic cats have been prepared after partial pancreatectomy. The number of animals cited in the tables varies according to the particular experiments performed. In producing pituitary-diabetes in the cat certain difficulties have been encountered. Excluding a few initial experiments, 19 permanently diabetic animals were obtained from 39 consecutive partially depancreatized cats treated with pituitary extract. This means that about 50 per cent of the animals became diabetic. The causes of failure in the 20 unsatisfactory animals have been classified as follows: 4 died of accidents before treatment with extract was begun; during treatment 3 developed peritonitis and 3 respiratory infections; 2 died of unknown causes and 8 became refractory to the extract, i.e., they lost their glycosuria while high doses of extract were being given.

Diets. During injection the cats were fed measured diets and the uneaten food was weighed to determine the daily intake. The daily diet was ordinarily 200 gm. of raw beef heart trimmed free of gross fat and supplemented by bone ash, yeast and cod liver oil. The use of other diets will be noted in describing the results. The total available glucose has been calculated (4) as 10.8 per cent of the beef fed. The average fat content was 5 per cent. Cats have been less upset than dogs by the injection of pituitary extract and more than 50 per cent ate the full diet offered throughout the period of injection. The percentage of the available glucose (D:N = 3.65) of the diet excreted in the urine has been used as a measure of the severity of the diabetes. In the cat this index of severity has varied more with changes in diet that it did in the dog (4). A glance at the figures for urinary glucose and available glucose in table 14 will

[1] The aid of Mr. J. C. Cole of Swift and Company in arranging for the collection of the glands is gratefully acknowledged.

illustrate this point. Nevertheless when the same diet has been used at different times this approximate measurement of severity has been useful.

Chemical. The methods of analysis have been the same as in previous reports (4, 8). Histological methods have been the same as those used in the dog (4).

RESULTS

A. *Observations During the Period of Injection of Anterior Pituitary Extract*

Amount of extract required to produce persistent diabetes. The fact that the cat with an intact pancreas cannot be made diabetic has been demonstrated as follows: Of 9 cats injected with large doses of extract (up to 20 cc. per day), 4 showed slight transitory glycosuria which disappeared in spite of continued injection. In the remaining animals, glycosuria never developed. This confirms Young's (2) observation that the normal cat is more resistant than the dog to diabetogenic pituitary extracts. This

TABLE 1. DURATION OF TREATMENT AND QUANTITY OF PITUITARY EXTRACT IN PRODUCTION OF PERMANENT DIABETES IN CATS

No. of cats		Weight of pancreatic remnant	Period[1] of pituitary treatment	Anterior pituitary gland[2]	
		gm.	days	total, gm.	gm./kg./day
19	Average	2.6	24	59	0.8
	Range	1.1–6.5	10–44	17–110	0.3–2.2

[1] From first to last injection inclusive. In many animals extract was omitted for a number of days in this period.

[2] See methods. The cats ranged from 2.3 to 4.1 (av. 3.3) kg. in weight.

situation is altered after partial pancreatectomy, as shown in table 1 which summarizes the quantity of extract and the duration of treatment required to produce persistent diabetes in 19 cats, and includes the weight of the pancreatic remnants. In some instances the weight of the pancreas was 0.5 to 1.0 gm. less than that at the time diabetes was produced, due to the removal of tissue for biopsy. The wide variation in the amount of pancreatic tissue in these animals was due to 3 cats. For the series tabulated the weight of the remnants averaged 2.6 ± 1.2 (S.D.) gm. (that of the normal cat pancreas being 5 to 7 gm.) and there was no apparent relation between the pancreatic mass and the quantity of extract required to produce diabetes. Although the removal of much pancreatic tissue at operation rendered the animals susceptible to pituitary extract, the small differences in the weight of their pancreatic remnants were not related to the readiness with which diabetes was produced. This was not surprising in view of the variation in island function among individual animals. This difference in the function of remnants of the same weight was illustrated by the fact that of 2 cats with 2.5 gm. of pancreas, one required 34 and the other 130 cc. of the same extract on the same diet, to produce permanent diabetes. In view of the wide range of island function possessed by these animals, it is not surprising that the duration of the period of injection as well as the amount of extract required should vary greatly. In this respect the partially depancreatized cats have exhibited individual differences comparable to those in dogs (4).

In spite of the effect of partial removal of the pancreas, the difference in the response of the normal dog and cat to anterior pituitary extract cannot be related to the gross size of the pancreas. The pancreatic weight, related either to body weight or to surface area, is essentially the same in the dog and cat as shown in table 2. The data

TABLE 2. RELATION OF PANCREATIC WEIGHT TO BODY WEIGHT AND SURFACE AREA (MEEH)

Species	No. of animals	Gm. per square meter		Percentage of body weight	
		Range	Average	Range	Average
Dog	14	25–86	48	0.13–0.38	0.22
Cat	5	24–41	30	0.16–0.32	0.21
Pig	5	26–61	44	0.17–0.28	0.22
Goat	5	27–52	40	0.10–0.21	0.15

on two other species are included for comparison. The figures for the dog have been calculated from Allen's data (9) and the other species have been observed in our laboratory. The fact that pancreatic weight does not reveal the amount of island tissue is clear when the effect of the anterior pituitary is the criterion. On the other hand, it is our impression from microscopic examination that the islands are larger and more numerous in the cat than in the dog.

As noted under methods, a small and constant daily dose of extract sufficed to maintain severe glycosuria in the partially depancreatized cat or dog in contrast to the large, progressively increasing doses required in the dog with intact pancreas (1, 4). As a result either of the smaller dose or of the cat's tolerance to intraperitoneal medication, the animals have eaten better than our dogs during the injection period. The advantage of this for certain types of experiment is clear. The gain in weight seen in the dog during the early days of injection with extract has seldom occurred in the partially depancreatized cat, presumably due to the rapid development of severe glycosuria.

Effect of dietary changes during pituitary extract. Even in animals with part of the pancreas removed so that glycosuria was readily produced by pituitary extract, it has been possible to demonstrate the prevention of this glycosuria by fasting. Similar findings were reported by Houssay (7) in the dog. Table 3 shows that the cat was originally without glycosuria, which developed in the usual manner when APE was given. After a rest period, the same dose of extract, during fasting, caused no glycosuria although it was still effective when food was given. Even in fasting animals, it is possible to produce some manifestations of diabetes if the pancreatic remnant is extremely small.

In the dog, Haist, Campbell and Best (10) have shown that, in addition to fasting, the administration of high-fat, low-carbohydrate diets or of insulin during extract treatment prevents the development of diabetes. We have confirmed this in the partially depancreatized cat, using isocaloric combinations of meat and fat (fig. 1). During these experiments, both S7 and S1 gained more than 200 gm. in weight during the fat

TABLE 3. RELATION OF FASTING TO THE GLYCOSURIA PRODUCED BY ANTERIOR PITUITARY EXTRACT
Cat 6. Partial pancreatectomy Sept. 19. Normal glucose tolerance Sept. 29.

Date	APE	Beef	Urine glucose	Date	APE	Beef	Urine glucose
	cc./day	gm./day	gm./day		cc./day	gm./day	gm./day
Sept. 30				Oct. 26	10	0	0
to	0	140	0	Oct. 27	10	0	0
Oct. 12				Oct. 28	10	0	0
Oct. 13	10	140	1.7	Oct. 29	10	140	1.9
Oct. 14	10	140	14.5	Oct. 30	10	140	6.9
Oct. 15	10	140	16.5	Oct. 31	10	140	10.6
Oct. 16[1]	10	140	15.5	Nov. 1	10	140	12.2

[1] Oct. 17 to 25: 140 gm. of beef daily; no extract; no glycosuria.

diet and an additional 200 gm. in the second 10 days on the full beef diet. Both fasting and high fat diets appear to be equally beneficial but the relative importance of reducing calories (fasting) and of reducing the glucogenic food (low protein diet) in these experiments requires further study.

Occurrence of acetonuria. In the first 18 permanently diabetic cats, examination for ketonuria during the injection of anterior pituitary extract resulted as follows: 6 had 4+, and 6 a trace to 1+ at some time during injection; 6 had no acetonuria. Ketonuria usually appeared in the last few days of injection as described in the dog (4). Four animals with transitory glycosuria while receiving extract had acetonuria before they

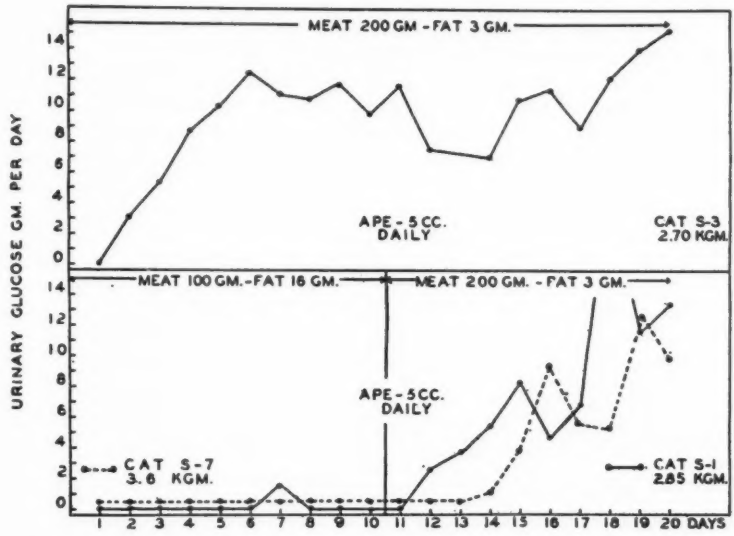

MEAT 200 GM - FAT 3 GM.
APE - 5 CC. DAILY
CAT S-3 2.70 KGM.
MEAT 100 GM. - FAT 16 GM.
MEAT 200 GM. - FAT 3 GM.
APE - 5 CC. DAILY
CAT S-7 3.6 KGM.
CAT S-1 2.85 KGM.
URINARY GLUCOSE GM. PER DAY
0 2 4 6 8 10 12 14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 DAYS

Fig. 1. Effect of diet on glycosuria from anterior pituitary extract. The days are numbered from the first injection of extract but the first diets received had been taken for a foreperiod of 6 days. Initial weights are given. On the 11th day (before 200 gm. beef) the blood sugars of S1 and S7 were 73 and 129 mg.% respectively.

became refractory to the extract. It appeared that the occurrence and degree of ketonuria had little bearing on the development of persisting diabetes. This does not exclude ketogenesis as a feature of the diabetogenic action of the pituitary extract because the formation of ketone bodies must be greatly increased before they are excreted (11). However, the early development of acidosis may hinder the production of permanent diabetes, as the animals then refuse food. The cessation of extract injection to allow their recovery is accompanied by the disappearance of glycosuria and the return of the blood sugar to normal. It is thus difficult or impossible to induce the sustained hyperglycemia and glycosuria which has always been associated with the production of permanent diabetes.

Rôle of the thyroid and adrenal glands in the action of pituitary extract. The mechanism by which the anterior pituitary exerts its diabetogenic effect is still obscure, but a few observations have been made which bear on this point. A comparison has been made between the glycosuric effect of thyroid and of pituitary extract in 2 partially depancreatized cats. In these animals the pancreatic remnants were small, the animals

TABLE 4. GLYCOSURIC ACTION OF THYROID AND ANTERIOR PITUITARY EXTRACT

Cat no.	Daily treatment	Maximum glycosuria	Period of treatment	Weight		Meat ingested
				Before	After	
		gm./day	days	kg.	kg.	gm./day
6	40 gm. thyroid	4.4	21	2.65	2.25	200
	5 cc. APE	9.6	2	2.70	—	200
	5 cc. APE	10.3	3	2.35	—	140
29	2 cc. APE	8.5	3	2.75	—	140
	40 gm. thyroid	0	21	2.60	2.35	200
	5 cc. APE	11.1	3	2.95	—	100

had slight, temporary glycosuria after operation and then became sugar-free. This suggested that they were very susceptible to metabolic stress. Thyroid was given as the raw gland minced with the beef feedings. The adequacy of the dosage was indicated by the loss of weight which occurred on a large diet of 200 gm. of meat (beef and thyroid) per day. Tachycardia, hunger and nervousness were also noted. Anterior pituitary extract was injected before or 15 days after thyroid treatment (table 4). *Cat 6* frequently had glycosuria of 2 to 4 gm. per day without any treatment. Although in the experiments cited the urine was sugar-free at the beginning of each treatment, the excretion of 4.4 gm. of glucose is not considered of great significance. It will be observed that during the last course of APE this animal's weight was not far above that at the end of thyroid treatment and that the diet was less than that during thyroid. The failure to develop glycosuria while receiving thyroid apparently was not due to the concomitant loss of weight since this did not hinder the glycosuric action of anterior pituitary. *Cat 29* was free of glycosuria except during APE treatment and the glycosuria occurred on diets smaller than the one used during thyroid administration. We have shown (12) that thyroidectomy is far less effective than

TABLE 5. RESPONSE OF PARTIALLY-DEPANCREATIZED CATS TO ANTERIOR PITUITARY EXTRACT BEFORE AND AFTER ADRENALECTOMY

Date	Meat	Urine glucose	APE[1]	Adrenal cortical extract	Date	Meat	Urine glucose	APE[1]	Adrenal cortical extract
	gm./day	gm./day	cc./day	cc./day		gm./day	gm./day	cc./day	cc./day
	Cat 6. Bilateral adrenalectomy 6/14					*Cat 29. Bilateral adrenalectomy 6/12*			
6/7	140	0	5		5/31	100	0	5	
6/8	140	7.5	5		6/1	100	6.1	5	
6/9	140	10.3	5		6/2	100	11.1	5	
6/16	50	0	5	5	6/16	20	0	5	5
6/17	127	0	5	5	6/17	73	0	5	5
6/18	90	1.8	5	5	6/18	100	0	5	5
6/19	100	3.0	5	5	6/19	100	0	5	5
	Cat 31. Bilateral adrenalectomy 4/6					*Cat 33. Bilateral adrenalectomy 5/25*			
3/28	200	0.5	5		5/18	200	0	5	
3/29	200	7.8	5		5/19	200	0	5	
3/30	200	15.5	5		5/20	200	9.1	5	
					5/21	200	12.8	5	
4/10	200	0	5	10					
4/11	200	0	5	10	5/29	200	0	5	10
4/12	200	1.3	5	10	5/30	200	0	5	10
4/13	200	2.7	5	10	5/31	200	2.1	5	10
					6/1	200	9.0	5	10

[1] Anterior pituitary extract given only on dates tabulated.

hypophysectomy in modifying pancreatic diabetes, and the same relation holds for the production of glycosuria when the respective glandular products are administered. Similar results have been reported in the dog (cit. in 12) and Allen (13) gave thyroid to 2 partially depancreatized cats with little effect. He noted that carbohydrate was far more powerful in producing hyperglycemia and glycosuria than thyroid. In like manner, anterior pituitary extract is far more powerful than thyroid.

In the totally depancreatized cat glycosuria could not be produced by pituitary extract in the absence of the adrenals (3), although some activity of the pituitary has been demonstrated in other experiments (14). However, when adrenalectomized and partially depancreatized cats were maintained with larger doses of cortical extract it was possible to evoke a small response by fresh saline pituitary extracts. These findings, given in table 5, show by the results of extirpation that the adrenal cortex is

TABLE 6. OBSERVATIONS ON CATS REFRACTORY TO ANTERIOR PITUITARY EXTRACT

Cat no.	Pancreatic remnant	Island lesions	Thyroid	Fasting metabolism after removal of pancreatic remnant	
				Urine glucose[1]	Ketonuria
	gm.			gm./kg./day	
R2	3.0	0	Normal	0.79	0
R4	3.5	++	Hyperplasia	0.77	0
R23	—	0	Normal	0.77	0
R13	2.1	0	Hyperplasia		
R18	2.0	++	Normal		
R22	2.2	0	Normal		
R26	4.3	0	Normal		
16 Depancreatized cats (12)				3.2	++++
19 Hypophysectomized-depancreatized cats (3)				0.7	0

[1] Average daily urinary glucose for first three postoperative days.

necessary for the full diabetogenic action of pituitary extract. The quantity (or potency) of cortical hormone would have to exceed that used in the above experiments before the grade of diabetes of the animal possessing its adrenals could be produced by replacement therapy in the adrenalectomized animal. The absence of diabetes in the adrenalectomized rat maintained on salt (14), the small response of adrenalectomized cats on ample life maintenance doses of cortical extract and the marked glycosuria of animals with normal adrenals all show the importance of these glands in the diabetogenic activity of the anterior pituitary. The findings in a species subject to permanent diabetes are thus in agreement with the extensive studies in the rat.

Refractory state. In connection with the injection of pituitary extract, certain findings in 7 animals which ceased to show glycosuria in spite of continued extract have been assembled (table 6). In 5 of 7 cats in this refractory state, the microscopic appearance of the thyroid was within normal; in only 2 was there the hyperplasia commonly seen during pituitary treatment (thyrotropic effect). Only 2 cats had lesions of the islands, and in both the glycosuria, although rapidly subsiding, had not disappeared as it had in the other animals. The adrenal and pituitary glands showed no abnormalities by routine stains.

Three of these refractory animals had the pancreatic remnants removed and were then fasted for 3 days to observe the diabetes which ensued. In table 6 it will be seen that after all of the pancreas was removed the glycosuria and ketonuria of these animals resembled that of hypophysectomized depancreatized cats and differed sharply from that of the standard depancreatized cat. In these animals the livers

showed no gross fatty change at autopsy. The results are similar to the antihormone phenomena produced in the dog (15) and indicate that pancreatic reserve or adaptation is not the sole explanation of the refractory state. The cause of the refractory state is unknown but the initial resistance to pituitary extract which is overcome by partial pancreatectomy and the subsequent refractory state which is apparent even after total pancreatectomy are clearly different conditions.

B. Studies During the Course of Diabetes after Treatment with Pituitary Extract

Degree of diabetes. The mildest form of diabetes has been that in which the animal has had no glycosuria on a large diet although there is hyperglycemia (120 to 150 mg. %) and a diabetic sugar tolerance curve. Two or three such animals have been ob-

TABLE 7. URINARY GLUCOSE AND NITROGEN EXCRETION OF FASTING DIABETIC CATS AFTER TERMINATION OF INJECTIONS

Cat no.	Period[1]	Weight	Average per day		Blood glucose	
			Urine glucose	Urine nitrogen		
	days	*kg.*	*gm.*	*gm.*	*day of period*	*mg. %*
5	33–35 (10)	1.7	0.1[2]	1.1		
18	31–33 (8)	2.5	4.1[2]	2.6		
R12 II	192–197 (16)[3]	4.5	1.5	—	2	258
R7	217–220 (176)	4.2	0[4]	—	3	157
R14	167–170 (146)	4.6	0.1[4]	2.4		

[1] Days of periods are inclusive and are counted from the first injection of APE. Number of days from last injection to beginning of period is in parentheses.
[2] Average excretion for last 2 days of period.
[3] The figure (16) refers to the second induction of diabetes—see tables 12 and 14.
[4] Fasting begun after a period of insulin treatment and when animal was without glycosuria.

tained. However, the majority of the cats have had moderately severe diabetes and almost every degree of diabetes has been encountered. The most severely diabetic animals have excreted 90 to 94 per cent of the available glucose of the meat diet and have lost weight at the rate of 50 gm. or more a day prior to treatment. The duration of this severe diabetes has not been observed as the animals were wanted for other experiments. The data on 5 cats illustrate these variations in the disease by the glucose excretion during fasting (table 7). It is clear that on fasting, these cats promptly became almost sugar free with the exception of *cat 18* which had severe diabetes. However, the glycosuria and nitrogen excretion of even this animal were slightly less than that of the average depancreatized cat. Six cats which excreted more than 90 per cent of the available glucose of the diet have been obtained but no other studies of the fasting metabolism have been made. They had only traces of ketonuria. The available evidence thus suggests that total urinary diabetes as described in the dog (4) has not occurred in the cat.

Duration of diabetes. Mild diabetes has been observed for periods of 6 to 9 months after stopping pituitary extract. There has been no doubt about the permanence of diabetes if the cats were otherwise in good health and ate regularly. In occasional animals, glycosuria, which had persisted for 2 to 3 weeks after the termination of extract, disappeared due to refusal to eat or to the healing of infection. Such animals have been excluded and the only modification of permanent diabetes to be considered herein is that produced by deliberate therapeutic measures. Figures 2 and 3 illustrate the duration of diabetes. In both, the period of extract treatment is included. These animals not only demonstrate the persistence of glycouria after stopping extract treatment but they serve as controls for the subsequent experiments on the recovery of

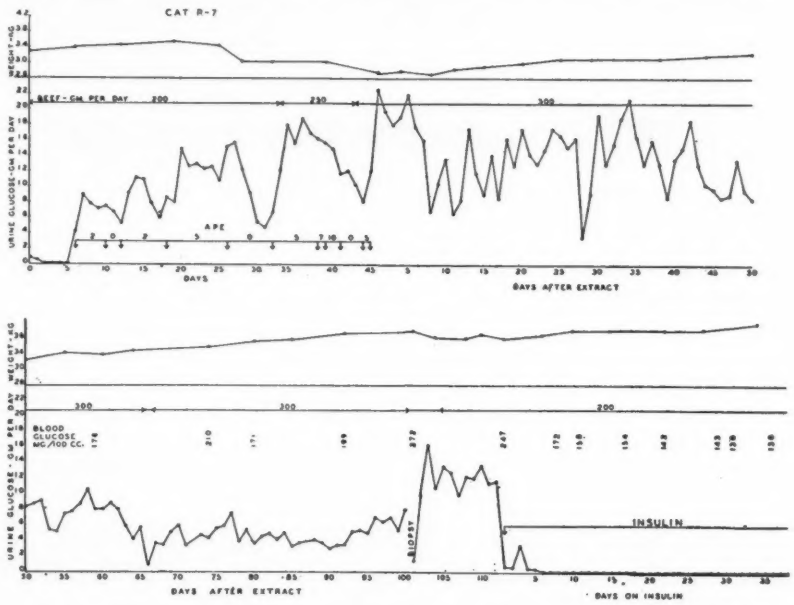


Fig. 2. Induction and course of diabetes in *cat R7*. The daily glycosuria is charted. Partial pancreatectomy had been performed 17 days before the first day on the chart. The figures above the arrows indicate the cc. of pituitary extract given daily for the periods designated. Autopsy was performed 84 days after the last point on the chart when the animal was still diabetic. Biopsy showed island atrophy.

early diabetes. Both cats were treated with insulin after 4 to 5 months of glycosuria and in the case of *R14* the diagram shows that diabetes was still present after insulin was discontinued. *Cat R7* lived 84 days beyond the last point on the chart. During this period insulin was stopped and the severity of its diabetes returned to the pre-insulin level. The relation between the duration of diabetes and the animal's ability to recover will be described below.

In connection with the duration of diabetes, its course after the cessation of pituitary extract may be noted. In figure 2 the glycosuria gradually declined and reached the level of a mild diabetes. The curve suggests an increasing glycosuria just before the first biopsy. In figure 3 the final glycosuria after insulin treatment was more severe than during the early periods of the disease and a progressive tendency is apparent. Six cats have been observed sufficiently to note the progress after extract. Of this

group, 4 have run a level course for 2, 7, 8 and 8 months and 2 have shown slight but definite progress in 30 and 170 days. These findings are clearly in contrast to the consistent progress of the post-injection diabetes in 4 dogs (4) in all of which the diabetes reached at least 85 per cent severity in 60 days after stopping extract. Permanent diabetes in the cat tends to be more stable and to progress much more slowly than it does in the dog.

Pancreatic pathology related to the course and to the response to treatment. Before presenting the results in feline diabetes, it is necessary to recall the previous studies in the dog (see also table 10).

In 1922, F. M. Allen (16) summarized the earlier work on the islands of Langerhans in experimental diabetes and presented his own monumental results. He used partially depan-

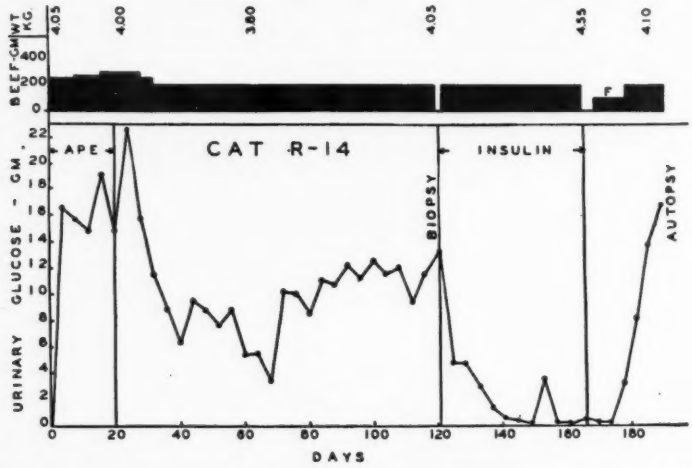


Fig. 3. INDUCTION AND COURSE OF DIABETES IN *cat* R14. Partial pancreatectomy was done 6 days before the first point on the chart. The curve shows the average daily glycosuria of consecutive 4 day periods, plotted on the last day of each period. *F* over the black area indicates a period on 100 gm. of beef and 10 gm. of lard. APE represents the total period of anterior pituitary treatment including trial omissions of extract. Biopsy showed island atrophy.

creatized diabetic dogs and showed that the islands developed hydropic degeneration within a week after sugar was found in the urine. This change progressed until the 4th or 6th week of glycosuria. Thereafter the islands of Langerhans underwent atrophy, becoming few and small. This change was not associated with any inflammatory reaction or fibrosis, except of course that which occasionally occurred as the gross result of surgery. In summarizing thus briefly Allen's description of the pathology of the islands, it must be remembered that the time required for atrophy to develop varied somewhat but its occurrence was inevitable. In these experiments, Allen also demonstrated the protective influence of dietary reduction. Our application of this principle in pituitary-diabetes is a natural consequence of Allen's work.

Copp and Barclay (17) demonstrated morphological recovery of hydropic islands in partially depancreatized diabetic dogs during treatment with insulin, as did Bowie (18). Although the hydropic islands were restored, recovery of the animal was not possible because the pancreatic remnant had been originally too small to support it. In their one animal with atrophy of the islands, no anatomical recovery followed insulin treatment.

In dogs, the lesion of the pancreatic islands in the permanent phase of pituitary-diabetes is *atrophy* (4, 19). Although other lesions have occurred this was the conspicuous feature in our 8 animals (4). This is understandable in the light of Allen's work because it usually takes

4 to 6 weeks of injection and another week or two after stopping pituitary injections to establish the presence of permanent diabetes. Insulin treatment has not resulted in recovery in pituitary-diabetic dogs with atrophy of the islands.

As already noted, Haist, Campbell and Best (10) have shown that fasting or the use of a high-fat, low-carbohydrate diet prevents the development of diabetes if used at the same time as the pituitary extract. In addition, the simultaneous injection of insulin protected the animals from the diabetogenic effect of the pituitary. These results, obtained by therapy before the development of atrophy of the islands, are in harmony with Allen's account of the pathology.

In the cat, as in the dog, diabetes has been associated with damage of the islands of Langerhans. Homans (20) demonstrated lesions in partially depancreatized diabetic

TABLE 8. RELATION OF HYPERGLYCEMIA AND GLYCOSURIA TO THE DEVELOPMENT OF EARLY ISLAND LESION IN TEMPORARY DIABETES

Cat no.	Glycosuria		Fasting blood glucose	Lesions		Remarks
	Degree	Duration[1]		Hydropic change	Degranulation	
		days	*mg. %*			
R15	slight	14	81	o	+	(1); previous blood sugar, 135
19	o	11	87	o	o	
R2	o	21	92	o	o	
82	o	9	94	o	o	
75	o	9	95	o	o	
R23	o	12	97	o	o	
P5	o	11	106	o	o	
R22	o	59	146	o	o	slight glycosuria, 50 days; none last 9 days
R4	slight	20	199	++	+	
P241	moderate	22	202	+++	+	(1)
S33	moderate	20	210	+	++	(1); blood sugar 125 7th day. 176 14th day
P237	slight	9	236	+	+	
R8	moderate	25	262	+++	+	
R19	severe	31	289	++++	o	
R11	moderate	12	295	++	++	
4	severe	5	574	o	+	

[1] In the absence of glycosuria the period of APE injection is given.
(1) Spontaneous diabetes after partial pancreatectomy—all others received APE.

cats after one month of glycosuria. In pituitary-diabetes in the cat, the islands showed hydropic degeneration for the first 3 months of diabetes, which included the period of injection and the early part of the permanent phase. After 4 months, the pancreatic islands had undergone atrophy, and, to a small extent, fibrosis. When permanently diabetic cats were treated with insulin before the 4th month of diabetes, it was found that the dose of insulin could be gradually decreased and finally stopped altogether. The animals then remained free of glycosuria, and hyperglycemia, and gained weight for periods as long as 3 months after the cessation of insulin. The islands of such cats have shown almost complete anatomical recovery. In contrast, animals in which treatment was not begun until 4 months after the onset of diabetes failed to recover and, as noted, their islands had become atrophic. With this brief introduction, the findings in tables 8 and 9 may be considered.

Table 8 shows the development of island lesions during the injection of pituitary extract. The animals have been listed according to the level of the blood sugar which

in most instances was established by a single determination made within a few days of taking the section. The blood sugar values tabulated do not tell the story for the whole period although they are in reasonable accord with the presence or degree of glycosuria which has been expressed qualitatively.

It is clear that island damage has not appeared in these animals except in association with hyperglycemia. In the presence of a high blood sugar there was a relation between its duration and the extent of the island damage. Thus, *cats 4* and *P237* with glycosuria for only 5 and 9 days had very minor lesions. In *cat S33*, earlier blood sugars had been 125 and 176 mg.% and the duration of severe hyperglycemia was shorter than the period of glycosuria. Three animals were not injected with pituitary extract but were spontaneously diabetic following partial pancreatectomy. They have been included because they conform to the findings of Allen (16) and Homans (20) as well as to the results in injected animals. Whether diabetes was caused by partial pancreatectomy or by pituitary extract, the presence of hyperglycemia and glycosuria has been associated with the development of island damage.

Table 9 summarizes the lesions of the pancreas in the permanent phase of diabetes. It includes cats in which biopsy or autopsy was performed prior to treatment with diet or insulin and also cats in which inadequate or late treatment was clearly ineffective. The animals in this table have been arranged in the order of the duration of glycosuria. From the results, it can be seen that at first the conspicuous lesion of pituitary-diabetes in the cat was hydropic degeneration. After approximately 3 months, atrophy of the islands, and, to a lesser degree, hyaline degeneration developed. The lesions thus summarized require further description.

As a rule, the pancreatic remnants were covered with light adhesions to the omentum and liver, beneath which the pancreatic tissue was grossly normal. The scarring of the pancreas itself was ordinarily very small and localized to the fibrosis about the ligature. In a few instances evident scarring of all or part of the remnant was apparent and biopsy material which was taken near the site of operation frequently showed scarring which was not seen when the remaining tissue was examined at autopsy. This scarring of known cause has been excluded in table 9 by considering only the lesions of islands surrounded by healthy acinar tissue. In several instances round cell infiltration was seen about the ducts and vessels and at times this has involved adjacent islands. No other lesions of the ducts, vessels or acinar tissue have been noted.

Number and size of islands. In the cat the islands of Langerhans have appeared to be larger and more numerous than in the dog. Their number and size remained normal throughout the stage of hydropic degeneration. At times the islands seemed slightly larger (swelling of cells?). As atrophy developed, only a few islands of normal size remained, the majority being very small. However, the number of island remnants was about the same as the number of islands normally present.

The individual island lesions in human diabetes mellitus have been considered at length by Warren (21) and a brief description of their occurrence in experimental diabetes will suffice. The earliest lesion of experimental diabetes, observed by all authors, has been *degranulation of the beta cells.* This is most accurately observed with special granule stains, but in our use of the term (table 8) a more advanced lesion has been considered, namely, the haziness and impairment of cytoplasmic structure visualized by the H. and E. stain. Degranulation was seen as the earliest stage of diabetes and as hydropic degeneration developed there was a period when both changes coexisted.

Hydropic degeneration was the characteristic lesion of the early stage of permanent diabetes and is illustrated in Plate I, figure *B*, and in text figure 4, C. Both degranulation and hydropic degeneration are reversible lesions (17) as will appear below. At the

time of early or incomplete recovery from hydropic degeneration, degranulation may be marked.

After diabetes had persisted for longer than 3 months the beta cells lost the power to recover. Although this sometimes occurred while the appearance was that of hydropic change, the shrinking of the nuclei was very great and some degree of atrophy of islands was also present.

TABLE 9. LESIONS OF THE ISLANDS IN PERMANENT PITUITARY-DIABETES RELATED TO DURATION OF THE DIABETES

Cat no.	Period[1] of insulin	Severity[2] of diabetes	Duration[3] of diabetes	Lesions of Islands			
				Hydropic degener.	Atrophy	Hyaline	Round cell infiltration
	days	%	*days*				
S21	0	70	20	++++	0	0	0
S7	1	100	23	++++	0	0	occasional
R17	13	85	30	++	+++	0	0
R25	0	94	30	++++	0	0	0
S22	0	54	30	++++	0	0	0
S6	6	63	35	++++	0	0	0
18	2	90	37	++++	0	0	0
R9	10	78	38	++++	0	0	0
S23	0	16	46	++	0	0	0
R20	0	66	50	+++	0	0	0
S3	0	13	55	++++	0	0	0
R11	0	65	56	+++	0	0	0
R27	17	53	71	++++	0	slight	0
R3	0	60	72	++++	0	0	0
R10	0	33	82	++++	0	0	0
5	0	56	82	++++	0	0	0
R14	0	63	120	+++	++++	0	0
S4	0	10	129	++++	+	slight	occasional
R7	0	20	140	++++	++	+	0
R21[4]	30	42	142	0	++++	0	0
R14	45	76[5]	190	+	+++	+	0
R7	49	37[5]	201	+	+++	++	0
S4	0	mild	227	0	+++	slight	occasional
R24	0	29	261	+	++++	0	0
R7	40	47	274	0	+++	+++	+
R10[6]		0	*463*	0	+	0	++
R12[6]		0	*315*	+	0	+	0

[1] Only the insulin-treated cats which were obviously diabetic at the time of section have been included.

[2] The severity of the diabetes is expressed as the percentage of the available glucose of the diet excreted in the urine during a period of several days preceding the biopsy or autopsy when not on insulin.

[3] Total duration of glycosuria during and after the injection of pituitary extract.

[4] Diabetes twice; cf. table 15.

[5] After insulin.

[6] Diabetes twice; functional recovery from insulin twice. See text under Repeated Diabetes. The duration is timed from the first pituitary extract given in the first episode of diabetes (italics).

Atrophy of the islands was the typical lesion of irreversible permanent diabetes in both dog and cat. It was characterized by a decrease in the size of the islands and in the number of islands which retained anything like normal appearance. The number of island rests or remnants may resemble the original island content of the pancreas. At this stage, many islands which were within normal size were found by suitable stains to be composed almost entirely of alpha cells. Atrophy of the islands was primarily atrophy of the beta cells although in the dog all traces of islands had sometimes vanished. Just as there has been a difference in the duration of hydropic change in the

cat and dog, so there has been a difference in the appearance of atrophy. This has been attributed to the change in the rate at which the disease advanced, rather than to any qualitative difference in the disease process. In figure 4, *B* and *A*, an attempt has been made to illustrate this difference in the appearance of atrophy in the dog and cat. In

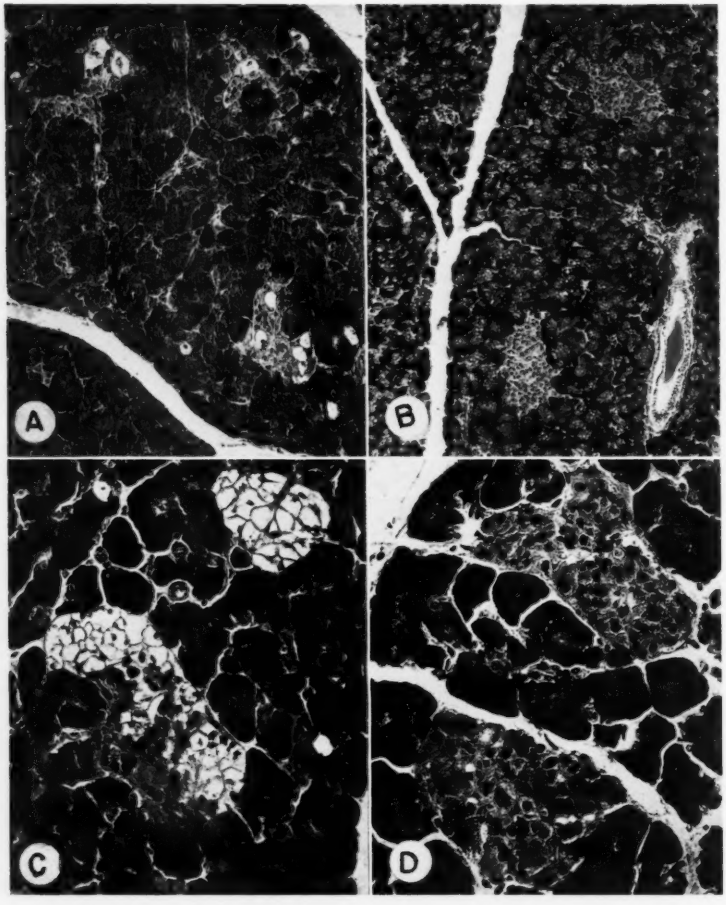


Fig. 4, *B*, Islands of *dog P30* (×92). *A*, Islands of *cat R14* (×92). The duration of diabetes was 202 and 190 days respectively. The dog was excreting 100 and the cat 76% of the available glucose of the diet. *C*, *cat R3*. Biopsy of pancreas after glycosuria for 72 days and before insulin treatment. ×240. *D*, *cat R3*. Pancreas, 66 days after the end of insulin treatment. ×240.

diabetes of about the same duration, (202 and 190 days), the islands of the dog (fig. 4, *B*) showed no hydropic change. They were few in number and those photographed might have been 'small normal' in size, but the Bensley stain showed that they were composed almost exclusively of alpha cells. In the cat (fig. 4, *A*) several island remnants are demonstrated composed not only of a few alpha cells but containing also 2 to 4 hydropic beta cells. The field was chosen as typical of the general microscopic survey

of the tissue. In one or two instances, small collections composed exclusively of alpha cells have been seen in the cat and the atrophy then has resembled that seen in the dog Hyaline degeneration and fibrosis were present in several animals observed for long periods (table 9) and the hyaline took the connective tissue stains. Hyaline has been conspicuous, fibroblasts few and no studies have been made to add to the past discussion of these lesions (21).

Finally, round cell infiltration of the islands has been noted. The cells were lymphocytes with occasional polymorphonuclear cells. Where this has been listed (table 9) the lesion was found in islands surrounded by normal acinar tissue suggesting the possibility that it was a type of primary island lesion. One cannot be certain of this, however, for in several of the animals with late diabetes the perivascular and perductal cellular infiltrations (pancreatitis) involved adjacent islands. An example of

TABLE 10. LESIONS OF PANCREATIC ISLANDS IN VARIOUS TYPES OF DIABETES

No.	Days of Glycosuria	Lesions					Source
		"Normal"	Hyaline	Fibrosis	Hydropic	Atrophy	
				Diabetes in man			
484	?	127	299	129	22	6	Autopsy data (20)
				Partial pancreatectomy in dogs			
10	0	10					(16)
19	5–50				15	4	(16)
6	20– 38				5	1	(17)
10	60–240				1	9	(16)
2	270–390					2	(22)
				Pituitary-diabetes in dogs			
6	5– 17				6		(4, 19, 23)[1]
5	177–401		3			2	(19)
8	49–418					8	(4)
				Partial pancreatectomy in cat			
2	29– 30				2		(20)
				Pituitary-diabetes in cat			
8	5– 31	1			7		from table 8[1]
16	20– 82				16	1	from table 9
5	120–274					5	

[1] During injection of anterior pituitary extract.

round cell infiltration is shown in Plate I, figure D. The association of lymphocytic collections and hyaline change in this figure was assigned to chance since it was not a common occurrence. On the other hand, some relation between pancreatitis and hyaline degeneration remains a distinct possibility. In all of the 7 animals with diabetes of 120 days or more in duration (table 9) there were occasional round cell collections about the interlobular ducts and vessels. In 4 of these there was some hyaline and in 3 occasional cellular collections were found in the islands. Two cats (*R21* and *R24*) which lacked both of these lesions had very marked atrophy. These observations, like Richardson's (19) suggest an association between pancreatitis and hyaline degeneration in the islands. The above details and the findings in table 9 have been briefly summarized in table 10 which relates the lesions of diabetes in the cat to those of canine and human diabetes reported by others. The selection of a single type of lesion in the dog and cat is a simplification of the above description.

In both early and late diabetes there was no obvious correlation between the extent of the lesions and the severity of the disease. In very mild diabetes a number of normal beta cells were usually found. On the other hand, severe hydropic degeneration or marked atrophy have been associated with wide variations in glycosuria. As

in the dog, the relation of the lesions to the physiological manifestations was highly approximate.

Other autopsy findings. Except in some cats in the refractory state, the animals which were receiving extract at the time of autopsy had marked hyperplasia of the thyroid. No changes have been seen in the pituitary or adrenal cortex, but very little material has been taken at the time when mitosis in the adrenals has been seen by others (23). In permanent diabetes (with or without recovery) the thyroid has appeared normal and this has also been the case in the spontaneous diabetes following extensive partial pancreatectomy. No changes have been found in the pituitary, gonads or adrenal cortex and the animals studied for 6 to 9 months have shown no lesions in the aorta and coronary arteries. The liver has been normal grossly and microscopically. Abnormalities of the urinary tract have occurred twice. *Cat R12* had hematuria for a few weeks before autopsy which revealed numerous crystalline bladder stones and normal kidneys. *Cat R24* had anorexia, polyuria and isosthenuria for the last month of its life and pyelonephritis was present at autopsy (serum urea N: 60 mg.%). The few liver glycogen and fatty acid determinations which have been made (table 11) agree with the findings in canine diabetes (4), if the severity of the diabetes is taken into account.

TABLE 11. GLYCOGEN AND FATTY ACID DETERMINATION IN PERMANENT DIABETES 24 HOURS AFTER LAST FOOD

Cat no.	Severity[1] of diabetes	Liver glycogen	Fatty Acids		Remarks
			Liver	Serum	
	%	%	%	%	
5	56	2.47	—	0.144	No insulin
18	90	2.48	2.05	3.45	24 hours after insulin
R14	76	2.74	2.48	0.312	No insulin
R24	30	—	2.15	0.105	No insulin

[1] Expressed as the percentage of the calculated available glucose of the diet excreted in the urine for 6 days without insulin therapy.

Recovery from Pituitary-Diabetes Following Early Insulin Treatment

The demonstration of the anatomical recovery from hydropic degeneration in partially depancreatized dogs (17, 18), implied that the same result was possible in pituitary-diabetes. This has been so and, in addition, functional recovery of the animals has occurred. The difference in the results from those in the dogs prepared in Allen's laboratory is attributed to the fact that diabetes followed partial pancreatectomy in his dogs only when so much pancreas had been removed that the animal could not utilize a full diet. In contrast, our partially depancreatized cats were not diabetic prior to pituitary treatment, so that after diabetes had been established the restoration of their originally adequate pancreas was associated with lasting recovery from the diabetes.

In considering the results in table 12 it is essential to recall the criteria of persistent diabetes described under methods, the permanence of diabetes when treatment was delayed (fig. 1, 2) and the total duration of diabetes (during and after APE) before treatment. In all of these animals, insulin was begun within the first 3 months of glycosuria. The recovered or non-diabetic state after stopping insulin was measured by: *a*), the absence of glycosuria; *b*), the normal level of blood glucose; and *c*), the ability to gain weight; all of these, *d*), on the same diet eaten prior to treatment. The first group

consisted of 5 animals which were undoubtedly diabetic before treatment and which were adequately observed after treatment. The second group was also satisfactory and has been distinguished because these animals recovered after a second induction of diabetes. The third group included two experiments subject to certain questions to be discussed which nevertheless added evidence of the value of therapy. Strictly speak-

TABLE 12. DIABETIC CATS RECOVERING AFTER INSULIN TREATMENT

Cat no.	Glycosuria		Severity[2] of diabetes	Duration of insulin therapy	Duration[3] of recovery	Increase[4] in body weight	Group
	During[1] injection of APE	Interval between APE and insulin					
	days	*days*	%	*days*	*days*	*gm.*	
R3	43 *1–43*	29 *44–72*	60	24 *73–96*	105 *97–201*	450	
R5	17 *2–18*	15 *19–33*	64	27 *34–60*	56 *61–116*	250	I
R10	24 *2–25*	57 *26–82*	43	20[5] *83–102*	62 *142–203*	200	
R12	10 *3–12*	12 *13–24*	63	32 *25–56*	113 *57–169*	250	
R28	19 *2–20*	7 *21–27*	90	23 *28–50*	25 *51–75*	100	
R10[6]	12 *204–215*	10 *216–225*	36	44 *226–269*	194 *270–463*	0	II
R12[6]	6 *170–175*	25 *176–200*	44	24 *201–224*	90 *225–315*	50	
R21	13 *1–13*	6 *14–19*	66	9 *20–28*	36 *29–64*	300	III
R25	14 *2–15*	15 *16–30*	94	31 *31–61*	5 *62–66*	—	

[1] In this and in other tables the figures in italics show the days from the first injection of APE and permit comparison between tables.

[2] Expressed as the percentage of the calculated available glucose of the diet excreted in the urine for 6 days prior to insulin therapy. For *R21* the previous 2 days were used.

[3] Non-diabetic period from cessation of insulin therapy to end of experiment.

[4] In the first 15 days after stopping insulin.

[5] Followed by dietary treatment. See table 14.

[6] Recovery from second episode of diabetes.

ing, there were 7 recoveries in 5 animals (*Groups I* and *II*). Figure 5 demonstrates graphically the course of one experiment.

The following individual animals require comment. A few days after stopping insulin, *cat R10*, in its first period of diabetes, had slight glycosuria (10%) which was controlled by reducing the diet to 100 gm. of beef (see table 14). It appears that this cat had moderately severe diabetes reduced by insulin to mild diabetes, which in turn was restored to normal by diet. This animal has been placed in table 12 as a recovery following insulin and in table 14 as a recovery following diet. In *cat R12*, in its second episode of pituitary-diabetes, a trial omission of insulin on the 18th day of the insulin treatment period was followed by the immediate return of glycosuria. The diet of *R12*

was 300 gm. of beef before and after the first episode of pituitary-diabetes. In *Group III, cat R21* had a nasal infection and was in such poor condition that insulin was given to save it. Its recovery after only 9 days on insulin throws doubt on the permanence of the diabetes and suggests that the subsidence of infection was of major importance in its cure. In this case, infection clouded the course of the diabetes, but the beneficial effect of insulin in a very ill diabetic animal was apparent. *Cat R25* had only 5 days of observation after stopping insulin, but the blood sugar was 82 mg. per cent at this time. This experiment was limited because of the development of an acute respiratory tract infection which was accompanied by a return of glycosuria and which led to the death of the animal in spite of the resumption of insulin treatment

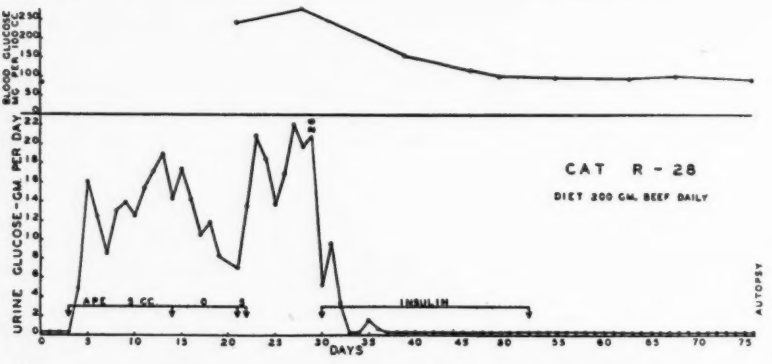


Fig. 5. Recovery following insulin treatment. *cat. R28.* Partial pancreatectomy performed 23 days before 1st injection of APE. Extract was given daily for the periods designated by the arrows. The dose of insulin was adjusted to control the diabetes and was gradually decreased in the days before its withdrawal. Compare figure 2.

and its continuance for 26 days. As glycosuria usually returned within 5 days in animals which had not recovered, this result is regarded as a significant improvement in severe diabetes due to insulin treatment. In all animals the increase in weight after stopping insulin was a continuation of the gain in weight which began during treatment. After its second recovery, *cat R10*, which did not gain in the first 2 weeks, added 1000 gm. to its weight in the next 6 months. The capacity to gain weight is of little significance in the case of those animals which had been gaining slightly before treatment (*R3, R5, R10-I*).[2] In the other experiments, the cats had lost from 100 to 450 gm. in the 2 weeks before insulin was begun and the ability to gain weight has been regarded as an index of improvement. The blood glucose levels before and after treatment are presented in table 13.

The insulin treatment consisted of 2 to 3 injections per day of both protamine and regular insulin,[3] the dosage being adjusted to keep the individual animal free of glycosuria. On this dosage the fasting blood sugar remained elevated for a week or more, and then became normal without increasing the insulin. It appeared that the action of insulin on the blood sugar level so assisted the islands of Langerhans that they could soon adjust for the irregularities of injection treatment. On the other hand, a sustained control of the blood sugar was necessary if the islands were to be fully restored, for in

[2] Roman numerals *I* and *II* following cat numbers in the text designate the first or second episode of diabetes.

[3] Insulin, Eli Lilly & Company, Indianapolis, Indiana.

TABLE 13. BLOOD SUGAR LEVELS BEFORE AND AFTER RECOVERY (9 CATS)

		Blood glucose				
		Fasting			Post prandial	
		Before treatment	After treatment		After treatment	
	No.	Range	No.	Range	No.	Range
		mg. %		mg. %		mg. %
Total	15	195–360	80	71–120	23	65–145
Average		274±9.6[1]		88±1.1		97±4.2

[1] S.D. of mean.

cat R10-II and *R12-I* the gradual omission of insulin on the 15th and 18th days of treatment after control had been well established led to the return of glycosuria. After further treatment it was possible to withdraw insulin and observe established recovery. Insulin was not stopped abruptly but was slowly reduced to 1 to 2 U a day before withdrawal.

The recovery following the early use of insulin has occurred in diabetes of widely varying degree including the most severe cases which we have encountered.

Response of Pituitary-Diabetes to Reduction in Diet

The glycosuria of 4 permanently diabetic cats on various diets is shown in table 14. In each instance the glycosuria on the full diet (200 gm. of beef) is given and the response to reduction of the meat, with or without added fat, is recorded. The first two animals (*Group A*) became sugar free as soon as the diet was lowered and after 26 and 24 days of this treatment the original diet was resumed with the usual evidence of recovery. This is illustrated in figure 6. The 2 animals in *Group B* were not controlled by the changes in diet and these animals did not recover with dietary restriction, for the diabetes returned to its original severity when the full diet was resumed. How-

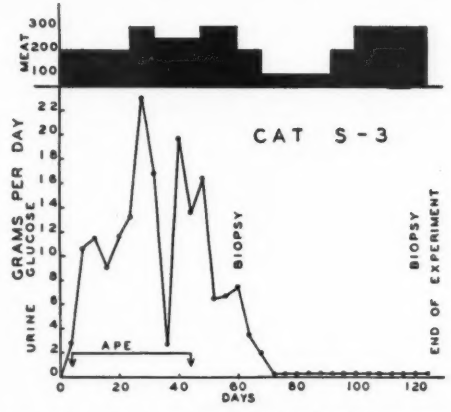


Fig. 6. RECOVERY FOLLOWING DIETARY TREATMENT. *Cat S3*. Partial pancreatectomy performed 18 days before 1st injection of APE. The arrows represent the total period of APE treatment including trial omissions of extract. The urine shows the average daily glycosuria of consecutive 4-day periods plotted on the last day of each period. The 1st pancreatic biopsy showed marked hydropic degeneration; at the 2nd biopsy there was only slight degranulation of the beta cells. Compare with figure 3.

ever, one of these, *R12*, subsequently recovered after insulin therapy, as has been noted (table 12, *Group II*). In addition to the diets recorded in Table 14, *Group B*, 3 animals with moderately severe diabetes (30–70%) have not been controlled upon reducing the diet to 100 gm. of meat without supplementary fat. The effect of maintaining the caloric value of the diet by means of fat cannot be discussed on the basis of such a small series of observations.

TABLE 14. RESPONSE OF DIABETIC CATS TO CHANGES IN DIET
No insulin given during these diet periods

Cat no.	Period[1]	Weight	Average per Day: Food B=beef L=lard	Average per Day: Urine glucose	Average per Day: Available glucose	Blood Glucose	
	day	*kg.*	*gm.*	*gm.*	*gm.*	*day of exp.*	*mg. %*
			A. Reduction in meat followed by recovery				
	111–115	2.95	B 200[2]	2.1	21.6	*111*	168
	(86)						
R10	*116–141*	3.05	B 100	0	10.8	*121*	95
	(91)						
	Recovery					*191*	87
	142–203	3.45	B 200	0	21.6	*201*	95
	61–66	2.85	B 200	2.7	21.6		
	(17)						
S3	*67–89*	3.05	B 100	0	10.8	*78*	88
	(23)						
	Recovery					*113*	86
	90–122	3.40	B 300	0	32.4	*116*	99
			B. Change in diet without recovery				
	182–174	4.80	B 200	9.5	21.6	*186*	277
	(6)						
R12							
II*	*188–191*	4.55	B 100	8.6	11.8		
	(12)		L 10				
	29–34	3.10	B 200	11.4	21.6	32	265
	(18)						
R27	*35–46*	3.10	B 100	3.8[3]	11.8	*46*	246
	(24)		L 10				
	47–56	3.00	B 50	1.7[3]	7.4	*56*	274
	(36)		L 20				

[1] Days of periods are inclusive and are counted from the first injection of pituitary extract. Number of days from last injection to beginning of period is in parentheses. Recovery periods are counted from the day when 200 gm. of beef or more was resumed.

[2] Diet begun at the end of a period of insulin treatment (*107th* day) when animal was without glycosuria. (See table 12).

[3] Average excretion last 4 days of period.

* Second induction of diabetes; this animal subsequently recovered after insulin treatment. (Table 12).

In these results the most striking fact was that only those animals with very mild diabetes (10 to 12% severity) were controlled and could recover under the reduction of diet. In this connection another animal (*S4*) with mild diabetes (10% severity) was treated after 4 months of glycosuria. Although the glycosuria was abolished for 24

days, it reappeared when the full diet was resumed. With diet as well as with insulin, delayed treatment failed to bring about recovery, as one would expect from the course of the island lesions. Furthermore, the effectiveness of dietary treatment in controlling diabetes is greatly limited by the severity of the disease in contradistinction to that of insulin. Thus far, adequate control of the manifestations of the disease has always preceded the sustained restoration of the islands.

Failure of Treatment in Pituitary-Diabetes

Nine cats have not recovered under treatment and the failures have been assigned to two causes. The first was faulty treatment and there were 4 cats that died from insulin reactions. The second cause was irreversible pathology and was exemplified by 4 cats treated after 3 months of diabetes (*R7*, *R14*, *R21-II* and *S4*) and one animal which, after biopsy, developed an abscess destroying all of the pancreatic remnant.

Condition of the Islands of Langerhans after Recovery

Three animals (*R3*, *R10-I* and *R25*) had biopsies taken before treatment leading to recovery. After demonstrating the consistency of the lesions of early diabetes in many animals it was not considered necessary to have such pretreatment biopsies to establish the presence of island lesions. Figure 4, *C* and *D*, show the islands before treatment and 66 days after stopping insulin in the same animal. After recovery, some of the nuclei of the beta cells appeared larger than normal, but when normal pancreas and tissue from the same animal as that in figure 4, *D* were mounted and stained together (Bensley-Mallory) the morphological restoration seemed to be complete. The other animals in *Group I*, table 12, had at autopsy complete recovery of hydropic degeneration but the beta cells showed traces of degranulation (H. and E. stain). The Bensley-Mallory stains gave good differentiation of alpha and beta cells, the latter with slight degranulation. This was also true of *cat R21*, *Group III*. *Cat R25* which developed a respiratory infection died during insulin treatment (94th day) and showed normal islands. The autopsies on *R10* and *R12*, *Group II*, which have been included in table 9, showed slight but distinct evidence of island damage after the second recovery. *Cat R12* was the only one which had traces of hyaline deposition after its first recovery and this is illustrated in Plate I, figure C. This section was selected because, although it shows striking recovery of hydropic degeneration, it also illustrates the minor structural changes which may persist after recovery. Although the anatomical restoration of hydropic islands was striking, it has not been perfect, and the significance of these residual findings will be considered in the discussion. At this point the story of the island changes in pituitary-diabetes may be reviewed by amplifying the legend of Plate I.

The numbers in italics indicate days after the first injection of pituitary extract and permit comparison with the tables. The prints were made from kodachrome photomicrographs. The following description of the plate does not include all the detail seen under the microscope.

In Plate I, figure *A* shows a section of an island of Langerhans of a normal cat. The alpha cells are recognizable by their light pink cytoplasm. Erythrocytes in capillaries are deep red. The beta cells are dull violet.

Figure *B* illustrates a section of an island from the pancreas of *cat 18*. Autopsy was after 37 days of glycosuria. The beta cells have undergone marked hydropic degeneration and are here seen as pale swollen cells with pyknotic nuclei and nearly complete replacement of cytoplasm. The irregular strands of cells, the cytoplasm of which still stains, are alpha cells. (Under the microscope these were easily identified and appeared undamaged.) This section was overstained to emphasize the absence of staining in the hydropic cells. This is the typical lesion of the first 3 months of diabetes (cf. table 9 and text fig. 8).

Figure C illustrates a pancreas section from *cat R12*, taken from a biopsy specimen on the 160th day (cf. table 12). This section was taken 104 days after stopping insulin when the animal had made a functional recovery. Alpha cells with pink cytoplasm can be distinguished from the erythrocytes in the capillaries by the presence of nuclei. Beta cells are dull violet, and are largely restored although the cell outlines are slightly less distinct than normal. The two small areas taking a blue stain are foci of hyaline degeneration. This section was mounted and stained on the same slide with that of figure A.

Figure *D* shows a pancreas section from *cat R7*, autopsied on the 274th day. No recovery occurred after delayed insulin treatment (see graph of text fig. 2). This island shows extensive hyaline deposition (bluish amorphous material) which has replaced approximately one-half of the cells. At the upper pole an area of round cell infiltration is present. This lesion is characteristic of late diabetes and is usually accompanied by atrophy as well as hyaline change.

Figure *E* illustrates a pancreas section from *dog P30*, autopsied after diabetes had been present for 202 days (during and after APE treatment). The island is very small and composed almost entirely of alpha cells. The magnification here is much greater than in the other figures in the Plate. This typical lesion of the permanent phase of diabetes in the dog is shown for comparison with Plate figure *D* and text figure 4*B*, the typical lesions in the cat.

Repeated Diabetes

After their recovery from pituitary-diabetes, which followed insulin treatment, three animals were made diabetic a second time by the injection of pituitary extract.

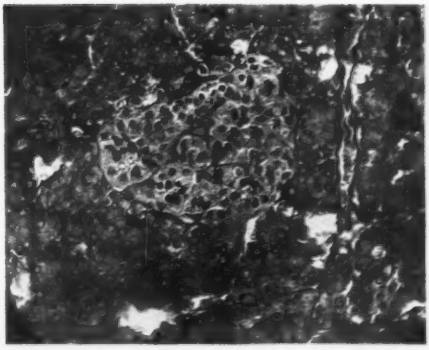
TABLE 15. COMPARISON OF FIRST AND SECOND INDUCTION OF DIABETES

Cat no.	Weight of pancreatic remnant	Period of[1] pituitary treatment	Dosage of[2] anterior pituitary glands	Diabetes
	gm.	*days*	*gm.*	
R10		25	60	I
		1–25		
	3.5	12	20	II
	463	*204–215*		
R12		12	33	I
		1–12		
	2.3	6	15	II
	315	*170–175*		
R21		13	36	I
		1–13		
	1.4	13	18	II
	142	*65–77*		

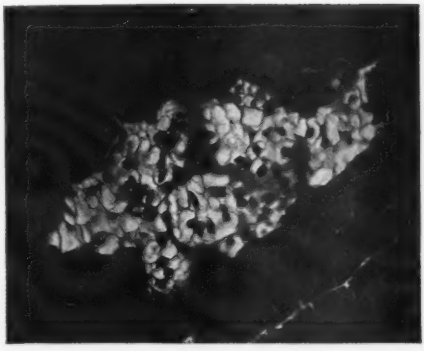
[1] From first to last injection, including trial omission of extract. Figures in italics indicate the number of days from the first injection of pituitary extract.
[2] See methods.

There was nothing notable in the character of the second bout of diabetes and in two animals a second functional recovery followed insulin treatment. In the third (*R21*), after unsuccessful insulin treatment, atrophy of the islands was found at autopsy (142nd day). Therefore, repetition of the island injury does not prevent recovery as long as irreversible lesions have not developed.

The most striking fact brought out by the repeated production of diabetes is presented in table 15 which shows that the quantity of extract was only half that originally needed to make the animal diabetic. The size of the pancreatic remnant at autopsy



A



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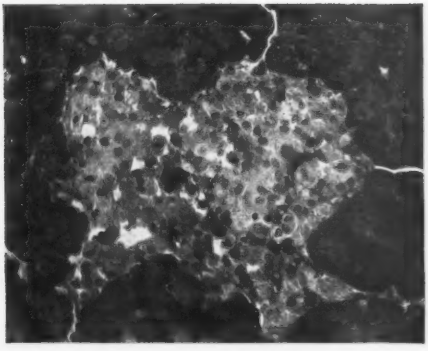

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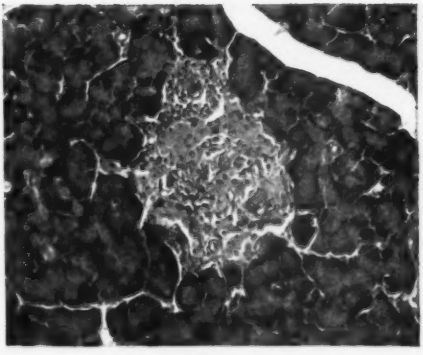
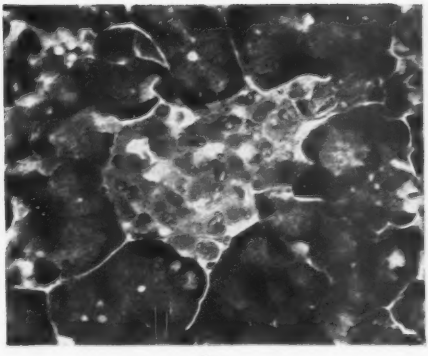


D



E

Plate I. PHOTOMICROGRAPHS OF ISLANDS OF LANGERHANS

Fig. A. From pancreas of a normal cat. (Bensley-Mallory stain. ×170).

Fig. B. From *cat 18*: early permanent diabetes with hydropic degeneration. (Bensley-Mallory stain. ×170).

Fig. C. From *cat R12*: early diabetes after treatment with insulin and functional recovery. Biopsy on *day* 160.(Bensley-Mallory stain. ×170).

Fig. D. From *cat R7*: diabetes for 274 days; showing hyaline degeneration. Autopsy on *day* 274. (Masson's stain. ×170).

Fig. E. From *dog P30*: diabetes for 202 days; showing atrophy of island. (Bensley-Mallory stain. ×300).

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made it unlikely that this was the result of biopsies performed between the 1st and 2nd episodes of diabetes, and there was no evidence that the extract used was more potent. From this experience with repeated diabetes, the conclusion has been reached that although satisfactory recovery had occurred, by the usual criteria, either the functional 'reserve' of the islands had not been restored to normal, or their susceptibility to injury was increased.

DISCUSSION

The major differences in the pituitary-diabetes of the partially depancreatized cat and the normal dog are as follows: *a*), technically, greater regularity of food intake and extract dosage has been possible in the cat and an economy of production and maintenance per diabetic animal has been effected; *b*), the course of mild or moderately severe diabetes is more stable and does not progress as it does in the dog (4); *c*), reversible hydropic degeneration persists for a longer time, thus permitting the study of the recovery of the islands under treatment early in the phase which, without treatment, would persist.

In pituitary-diabetes the rôle of the thyroid in the diabetogenic effect must be relatively small as indicated by the following facts: *a*), thyroidectomy has little effect on pancreatic diabetes (12); *b*), the administration of thyroid to partially depancreatized animals is relatively ineffective in inducing diabetes when compared with pituitary extract; *c*), thyroid hyperplasia, although present during pituitary treatment, is not a necessary accompaniment of diabetes as it does not occur when diabetes spontaneously follows partial pancreatectomy; *d*), purified thyrotropic hormone fails to produce diabetes, at least from present evidence (24); *e*), pituitary extract is diabetogenic in thyroidectomized dogs (25).

The rôle of the adrenal cortex in experimental diabetes has been fully reviewed (14) and its importance in the production of pituitary-diabetes in the cat is indicated in the single type of experiment shown in table 5.

The nature of recovery. The nature of the recovery of these animals requires further discussion. From what has been said of the course and pathology of the disease in the cat and dog, it is clear that the reversal of hydropic degeneration by Copp and Barclay (17), the prevention of this lesion by Haist, Campbell and Best (10) and the restoration of the islands in our cats have one feature in common; *i.e.*, all experiments have forestalled the development of irreversible types of island damage. We have seen no evidence of the regeneration of new island cells. The influence of the species on the character and rate of progress of the pathological process is related to the period within which effective treatment can be initiated. Furthermore, emphasis upon species differences restrains hasty inferences relating to human diabetes. The criteria of recovery may be reviewed under two headings. *a*), *The duration of recovery* which we have studied has been limited by laboratory conditions. The recoveries in our animals persisting from 5 days (*R*25) to 6 months (*R*10) are satisfactory as acute experiments, although really permanent recovery would be that which persisted for the full life expectancy of the species studied. The observed duration of improvement in our animals is quite sufficient to indicate the valuable effect of early treatment. Most remissions of the disease in patients (26) are followed by the return of diabetes many years later, reminding us that the observed duration of recovery in our animals does not complete the story. *b*), *The quality of recovery.* The physiological criteria of normal fasting and post prandial blood sugars, freedom from glycosuria, general health and an increase in weight on a standard diet have been noted. Two observations have served to show that these apparently excellent recoveries have not been perfect. The first has been the presence of minor lesions in the islands of cats examined after successful

treatment. These have consisted of slight degranulation with occasional enlargement of the nuclei of the beta cells, and the presence of very slight chronic lesions (hyaline degeneration and round-cell infiltration) in two instances. The second finding, indicative of limited recovery, has been the increased susceptibility of 3 recovered cats to a second course of pituitary extract. Infection frequently produces such an exacerbation of diabetes after remissions in man and it shortened the experiment in *R25*. Spontaneous infection is too uncontrollable to permit the measurement of susceptibility recorded in table 15. It is possible that a more prolonged period of treatment might lead to still better improvement. However, the improvement observed in our animals provides further incentive for the study of the early stages of human diabetes mellitus.

The mechanism by which insulin or diet produces recovery may be suggested, at least as a tentative hypothesis. When insulin is given, its beneficial effect might well be due to something other than the control of hyperglycemia. In any case, the picture of recovery after insulin or diet is essentially that of a transition from inadequate to adequate insulin brought about by the administration of insulin or by reducing the need for insulin to fit the supply available in the mildly diabetic animal. In an effort to distinguish between the effect of adequate insulin and that of reduction of hyperglycemia we have recently treated diabetic cats with phlorhizin for 2 to 3 weeks without undernutrition (27). This has resulted in a prompt and sustained lowering of the blood sugar, presumably by its action on the renal threshold. Morphological and functional recovery from the diabetes has followed the cessation of phlorhizin. It may be that the action of phlorhizin and that of insulin coincide in some respect other than the lowering of the blood sugar; but at present this is the only common factor which we can offer to explain the recovery of the hydropic islands under the several forms of therapy which have proved effective.

The above statement raises the question of the rôle of hyperglycemia in pituitary-diabetes. In the stage of extract treatment, pituitary-diabetes is a process which may depend on the altered state of several endocrine glands resulting in the disordered metabolism. However with respect to the pathogenesis of island lesions and subsequent permanent diabetes, we have been impressed by the possible importance of the elevation of the level of blood glucose. As a working hypothesis, the development of the permanent phase of diabetes may be stated as follows. The administration of pituitary extract results in: *a*), the production and maintenance of hyperglycemia, probably by its extra-pancreatic action; and *b*), the damage to the pancreatic islands associated with this hyperglycemia, be it as cause or effect. The first of these has been competently discussed (28, 29). A discussion of the possibility that injury to the islands may result from hyperglycemia must take account of two facts. These are the diminished liberation (30) and content (31) of insulin induced by pituitary extract and the increased production of insulin which follows elevation of the blood sugar in the normal animal (32, 33). The results of Best, Campbell and Haist (31) provide the most recent correlation between pancreatic insulin, blood sugar levels and island lesions. Regardless of the changes in insulin content, no permanent or irreversible damage to the islands has been produced by anterior pituitary treatment except when there is concomitant hyperglycemia. In addition, in the case of the cat, the high blood sugar persists after stopping pituitary extract even though the pancreatic lesions are still reversible. This suggests that hyperglycemia maintains the island damage (a vicious cycle). Stated in brief, the hypothesis that the elevation of the blood glucose is an important factor in the causation of island pathology is supported by the following general observations. *a*), Partial pancreatectomy (16) or pituitary extract do not cause island lesions in the absence of hyperglycemia, and, *b*), each leads to hydropic degeneration after hyperglycemia has been present for a week or two. *c*), The elevation of

the blood sugar by perfusion has resulted in early lesions of the islands (34). *d*), The islands are restored by various types of treatment which return the blood sugar to normal levels.

The above hypothesis rests upon an association of facts but does not include all the abnormalities of diabetes. As stated above the early hyperglycemia may be associated with island damage as cause or as result. One item of fact and one of theory may be cited to emphasize the need for an open mind concerning the pathogenesis of diabetes. *a*), The pancreatic remnants of a few diabetic rats prepared by Drs. C. N. H. Long in New Haven and D. J. Ingle in this laboratory have been examined. Although these animals had had marked hyperglycemia for periods of 2 weeks to several months, they showed almost no hydropic change, and atrophy of the islands was not conclusively demonstrated. Such findings have been reported by Friedman and Marble (35). The state of the islands in the diabetic rat remains to be brought into line with the course of events in the dog and cat. *b*), The supposition that hyperglycemia damages the islands is made without knowledge of the mechanism involved. The theory of 'strain' (10, 16, 19) has been challenged largely because of its vagueness.

In conclusion, we have noted the fact that pancreatic lesions are produced by partial pancreatectomy or pituitary extract only in association with hyperglycemia. In like manner, the recovery of the islands is coincident with the return of the blood sugar to normal levels prior to irreversible damage to the islands, whether this is accomplished by insulin, dietary treatment or phlorhizin (27). These findings support the hypothesis that the level of blood glucose is not only an index of the regulation of carbohydrate metabolism, but also that it has, directly or indirectly, an influence upon the islands of Langerhans. This influence is pathogenic when the blood sugar is high and beneficial when hyperglycemia is controlled, provided the lesions are not irreversible.

SUMMARY

Although the normal cat has not been made diabetic by the injection of crude saline anterior pituitary extract, the partially depancreatized cat without spontaneous diabetes has been satisfactory for the study of certain aspects of pituitary-diabetes.

A. *During the administration of extract.* 1. The glycosuric action of the anterior pituitary has been prevented by fasting and by altering the proportions of fat and protein in adequate isocaloric diets.

2. The response to pituitary extract has been contrasted with that to thyroid administration. The diabetogenic action of APE has been greatly modified by adrenalectomy. The findings in the cat were in accord with the present knowledge of the rôle of these glands in metabolism.

3. The initial resistance of the normal cat to pituitary extract appears to depend on the size of the island reserve. During extract treatment cats have occasionally developed a refractory state in which the removal of all the pancreas results in a Houssay type of diabetes.

B. Diabetes persisting after the termination of pituitary extract treatment. 1. The diabetes has varied widely in its severity but has not equalled that of total pancreatectomy.

2. The persistence of the diabetes has been observed for periods up to 9 months.

3. The course of diabetes was not uniform, some animals showing slow progression, others remarkable stability of the disease.

4. Hydropic degeneration of the beta cells was the characteristic lesion of the islands of Langerhans for the first 3 months of glycosuria.

5. After 3 months of glycosuria atrophy of the islands was the conspicuous lesion.

In addition, hyaline degeneration, fibrosis and lymphocytic infiltration of the islands were occasionally seen.

C. *The response of permanent diabetes to treatment.* 1. Treatment of the diabetes begun within the first 3 months has resulted in morphological restoration of the islands and functional recovery of the animals which was maintained after therapy was discontinued.

2. Recovery from early diabetes has followed *a*), reduction in diet only when the diabetes was very mild and *b*), treatment with insulin regardless of the severity of the disease.

3. When treatment by diet or insulin was delayed until after 3 months of diabetes, recovery did not occur. At this time, as noted, the islands had become atrophic.

Three diabetic cats which had recovered after insulin had been discontinued were again made diabetic by pituitary extract. In 2 cases a second recovery followed the cessation of insulin treatment.

The mechanism of the production and reversal of island damage has been discussed and the hypothesis that the level of blood glucose may be an inportant factor in the pathogenesis and in the recovery of hydropic islands has been considered.

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A STUDY ON THE SPECIFICITY OF THE ANTI-INSULIN EFFECT

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IT WAS FIRST SUGGESTED by Jensen and Grattan (1) that the anti-insulin (glycotropic[2]) effect of the anterior pituitary may be attributed to the adrenocorticotropic principle of that gland and is mediated through the adrenal cortex. Grattan and Jensen (4) found that apparently only those principles of the adrenal cortex substituted in ring 3 (keto- or hydroxy- group) are capable of inhibiting insulin hypoglycemia. This latter finding is in agreement with the observations of various other investigators (5–9) that apparently only those adrenal cortical principles, which contain either a keto- or hydroxy-group at C_{11} exert a significant influence on carbohydrate metabolism. Grattan and Jensen (4) suggested that the anti-insulin response produced by the adrenocorticotropic hormone and the corticosterone-like principles is probably due to the ability of these substances to induce the formation of glucose from non-carbohydrate sources with its subsequent deposition as liver glycogen. Hartman and his associates (10) have likewise concluded that the increased resistance to insulin of normal fasted mice after cortin injection is apparently due to gluconeogenesis, since both the blood sugar and liver glycogen were found to be elevated. Grattan, Jensen, and Ingle (11) recently reported that in adrenalectomized mice the administration of the adrenocorticotropic hormone of the anterior pituitary does not inhibit insulin hypoglycemia and does not give rise to an increase in liver glycogen. On the other hand the administration of corticosterone was found to be just as effective in operated as in normal animals. These various findings would seem to support the following assumptions: *a*), that the adrenocorticotropic factor of the anterior pituitary is responsible for the anti-insulin effect of that gland; *b*) that this response is mediated through the adrenal cortex; *c*) that gluconeogenesis is stimulated, resulting in an increase of liver glycogen; and *d*) ,that the increased liver glycogen stores explain the resistance of the animal to subsequent insulin injections.

Since it is known that the administration of a number of substances can modify carobohydrate metabolism and also insulin resistance, it is desirable to differentiate the effects according to the mechanism involved. As a specific effect we consider only that resistance to insulin which is brought about by an increased formation of glucose from noncarbohydrate sources and its subsequent deposition as liver glycogen. A non-specific protection against insulin administration may simply be due to a redistribution of carbohydrate stores rather than to a new formation of carbohydrates. Thus the anti-insulin effect of any substance eliciting an increased secretion of epinephrine[3] for instance, would be considered as non-specific, since it produces

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[2] Young has proposed the term "glycotropic" for the anti-insulin response of the anterior pituitary (2, 3).

[3] The secretion of the posterior lobe of the pituitary may also be considered in this connection since it is known to exert an anti-insulin effect.

only a redistribution and not a new formation of carbohydrate stores. The important difference between the two types of anti-insulin effect, therefore, lies in the formation of glucose from non-carbohydrate sources and its deposition as liver glycogen induced after administration of the test substance at the onset of a 6-hour fast. We feel that this difference in behavior has to be considered in testing for the possible presence of the anti-insulin factor of the anterior pituitary in blood, urine, and different organs. Various investigators (12–17) have reported the possible presence of an anti-insulin principle in urine and blood, especially that of diabetic patients. Their findings, however, are not in complete accord, and no studies on the influence of the test preparation on the formation of liver glycogen have been recorded. We, therefore, have studied different urine preparations with regard to their effect on blood sugar, insulin sensitivity, and liver glycogen, A preparation obtained from the urine of dogs injected previously with freshly prepared anterior pituitary extract was also studied for anti-insulin activity, since Himsworth and Kerr (16) have reported the presence of an anti-insulin factor in the urine of rabbits treated in a similar way.

EXPERIMENTAL PROCEDURE AND RESULTS

The following preparations were studied for their effect on blood sugar, insulin hypoglycemia, and liver glycogen.

Preparation A, gonadotropin from menopause urine, 4 units per mg.[4]

Preparation B, gonadotropin from normal male urine, 2 units per mg.[4]

Preparation C, gonadotropin from human pregnancy urine, 200 units per mg.[4]

Preparation D, crude beef anterior pituitary extract, obtained by grinding freshly dissected anterior lobes with sand in ice-cold saline (1 liter of saline per 370 gm. of glands). The mixture was adjusted to pH 8.5 and allowed to stand in the ice box overnight. It was then centrifuged and saline added to the supernatant fluid to give a final volume of 1110 cc. and adjusted to pH 8.5 (3 cc. of solution corresponding to 1 gm. of beef anterior pituitary).

Preparation E, 'anti-insulin factor,' prepared from dog urine according to the technic of Himsworth and Kerr (16). Two dogs (fox terrier) weighing 6.5 and 7 kg., respectively, received 3 subcutaneous injections of 36 cc. each of a freshly prepared extract of beef anterior pituitary as described above (preparation *D*) during a 24-hour period. The urine passed during that period was collected under toluene. It was found to give a strongly positive reaction for glucose. The anti-insulin factor was precipitated from the pooled urine (800 cc.) with benzoic acid, the precipitate collected, extracted with acetone, and the acetone-insoluble material removed and dried, yielding 470 mg. of solids. A similar precipitate was prepared from pooled urine specimens of the dogs prior to treatment with anterior pituitary extract.

Effect of urinary gonadotropins (preparations A, B and C) on the blood sugar of rabbits. Rabbits which had been fasted for 24 hours, were injected intraperitoneally with 15 mg. of the test preparation in 1 cc. of water per animal. The blood sugar values were determined at certain intervals during the next 6 hours. The results are illustrated in figure 1.

Anti-insulin effect of urinary gonadotropins (preparations A and B) in rabbits. Fifteen mg. of either gonadotropins *A* or *B* in 1 cc. of water were injected twice, subcutaneously, 7½ and 26 hours after onset of the fast (total dose 30 mg. per rabbit). Three hours after the last injection, 0.5 U of insulin per 2 kg. rabbit was given intra-

[4] The various urinary gonadotropic preparations (Gamone, from menopause urine; Prospermine, from normal male urine; and Follutein, from human pregnancy urine) were kindly supplied to us by Dr. J. S. De Frates, Biological Laboratories, E. R. Squibb and Sons, New Brunswick, New Jersey.

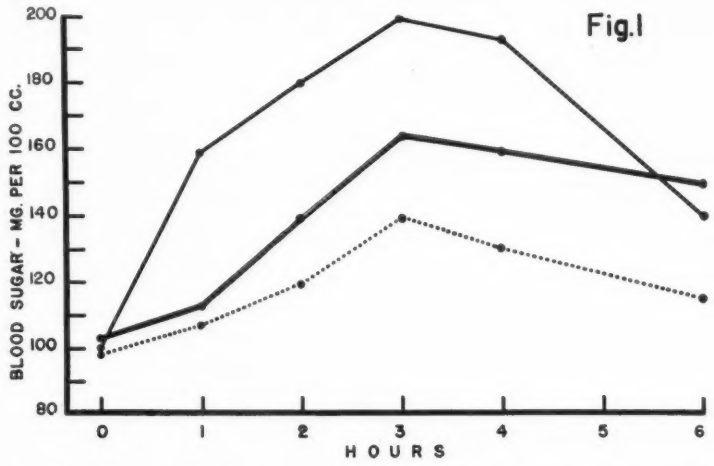

Fig. 1. Blood sugar curves of rabbits on intraperitoneal injection of 15 mg. of the test preparation in 1 cc. of water per animal. The curves represent the composite results obtained in 12 animals. Solid single line, gonadotropin from menopause urine, *preparation A;* double line, gonadotropin from human pregnancy urine, *preparation C;* broken line, gonadotropin from normal male urine, *preparation B.*

venously. Blood sugar determinations were made just prior to the insulin administration and at certain intervals during the next 2½ hours. Control rabbits received 1 cc. of physiologic saline solution per animal in place of the urinary preparation. The results are shown in figure 2.

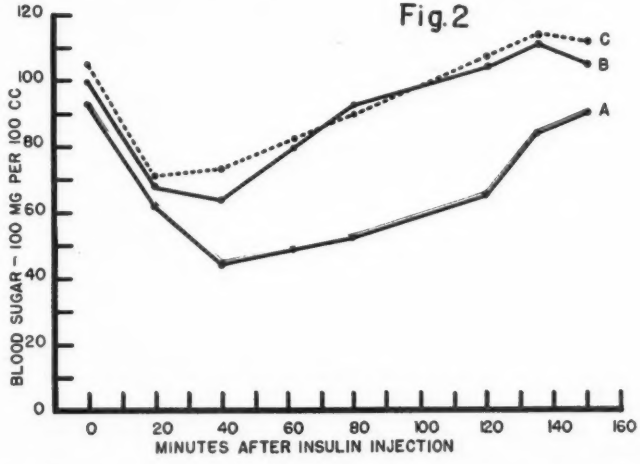

Fig. 2. Influence of urinary gonadotropins on the hypoglycemic action of insulin in the rabbit. A, Blood sugar response to intravenous injection of 0.5 u per rabbit, after 29-hour fast period. *B,* Response to intravenous injection of 0.5 u of insulin in rabbits which received two 15 mg. subcutaneous injections of *preparation* A during the preliminary 29-hour fast. C, Response to intravenous injection of 0.5 u of insulin in rabbits which received two 15 mg. subcutaneous injections of *preparation B* during the preliminary 29-hour fast. Each curve shows the composite of the results obtained in four animals.

Anti-insulin effect in mice. The procedure followed in determining the anti-insulin effect of the various preparations in mice was the same as that previously employed by Jensen and Grattan (1). The preparations tested and their influence on insulin resistance are given in table 1.

TABLE 1. ANTI-INSULIN TESTS IN MICE

Preparation injected[1]	Insulin dose per kg.	Total number of animals	Convulsions	
	U		no.	%
0.5 cc. saline (control)[2]	1.5	20	15	75
5.0 mg. *preparation A* (menopause urine)[2]	1.5	34	14	41
5.0 mg. *preparation B* (normal male urine)[2]	1.5	33	7	21
0.4 cc. saline (PH 8.5) (control)	2.0	15	12	80
125.0 mg. fresh beef anterior pituitary (0.4 cc.) *preparation D*	2.0	25	4	16
Uninjected controls	1.5	25	18	72
10.0 mg. preparation from normal dog urine	1.5	12	8	67
Uninjected controls	2.0	51	48	94
5.0 mg. *preparation E* from urine of dogs previously receiving beef anterior pituitary	2.0	22	11	50
10.0 mg. *preparation E*	2.0	15	5	33
0.2 cc. peanut oil control[2]	1.5	30	24	80
0.04 mg. epinephrine (in 0.2 cc. peanut oil)[2]	1.5	30	15	50

[1] All preparations were injected subcutaneously in 0.5 cc. volume unless otherwise stated.
[2] Total dose administered in 2 injections at 0 and 3 hours from onset of 6-hour fast.

Effect on liver glycogen in mice. The method employed for the determination of liver glycogen was the same as previously described by Grattan and Jensen (4). The preparations tested and the results obtained are shown in table 2.

TABLE 2. LIVER GLYCOGEN TESTS IN MICE

Preparation injected[1]	Number of animals	Average wt. of animals at onset of fast	Average wt. loss per animal during fast	Average liver wt. per animal	Liver glycogen
		gm.	gm.	gm.	mg. %
Controls	50	20.8	0.65	1.14	510
5.0 mg. *preparation A*[2]	20	21.3	0.25	1.24	495
5.0 mg. *preparation B*[2]	20	21.0	0.15	1.26	595
0.08 mg. epinephrine (in 0.2 cc. peanut oil)[2]	20	21.2	0.70	1.13	428
125.0 mg. fresh beef anterior pituitary (0.4 cc.), *preparation D*	28	20.9	0.61	1.25	1390
10.0 mg. *preparation E*, from urine of dogs previously treated with anterior pituitary extract	18	20.7	0.39	1.23	564

[1] All preparations were injected subcutaneously in 0.5 cc. volume unless otherwise stated.
[2] Total dose administered in 2 injections at 0 and 3 hours from onset of 6-hour fast.

DISCUSSION

It is obvious from the various results recorded that apparently a variety of substances obtained from urine under different experimental conditions are capable of

producing an elevation of blood sugar and a certain degree of anti-insulin response in normal rabbits and mice. It should be pointed out that the influence of the different urinary gonadotropic preparations on blood sugar and insulin hypoglycemia is apparently not related to their gonadotropic potency but is probably due to certain impurities present. Preparations obtained from dog urine before and after injection of the animal with pituitary extract show a definite difference in their degree of anti-insulin response. In normal mice, the various urinary preparations were found to produce no increase of the liver glycogen under experimental conditions in which crude anterior pituitary extract, adrenocorticotropic preparations and certain adrenocortical principles will not only produce an anti-insulin effect but also a definite increase in the deposition of liver glycogen (1, 4).

Our findings show that substances producing an anti-insulin response may be differentiated by their influence on liver glycogen; they support our contention that a distinction should be made between a specific and nonspecific anti-insulin effect. The nonspecific anti-insulin response can be produced by such substances (e.g., adrenal medulla and posterior pituitary) which may bring about only a redistribution of the carbohydrate stores in the test animal without any formation of new carbohydrate. A specific anti-insulin response is elicited by such substances which are capable of producing an increase in the deposition of liver glycogen by forming glucose from non-carbohydrate sources.

SUMMARY

It has been shown that various urinary preparations produced an elevation in blood sugar and an anti-insulin effect in normal rabbits and mice. They were found, however, not to increase the glycogen content of the liver under similar experimental conditions.

In contrast, certain endocrine principles, eliciting an anti-insulin effect, have been found to promote the deposition of liver glycogen (1, 4).

A distinction between a specific and a nonspecific anti-insulin (glycotropic) effect has been proposed.

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CREATINE-CREATININE METABOLISM AND THE HORMONES

VIII. THE EFFECT OF INCUBATION OF DIFFERENT TISSUES WITH AND WITHOUT PITOCIN, PITRESSIN AND THYROXIN UPON THE CREATINE-CREATININE EQUILIBRIUM IN VITRO

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IT IS WELL KNOWN that during thyrotoxicosis or after administration of thyroxin there is an increase in creatine excretion followed by a retention of creatinine. Pitressin and pitocin cause a retention of the creatinine formed in the body from amino acid metabolism followed by a quantitative increase in creatine excretion. It is, therefore, possible that creatinine may be the mother substance of creatine under these conditions. The experiments performed during the present study were designed to investigate this possibility. A search for enzymes capable of acting on creatine and creatinine was also made.

METHODS

Creatinine, free from creatine, was dissolved in a phosphate buffer of pH 7 in physiological saline to contain 1 mg. of creatinine per cc. Fifty mg. of creatinine was used in the first four studies and 100 mg. in the last study. To these solutions different amounts of rat tissues and the hormone solutions were added in the amounts shown in the tables and chart. In one study 100 mg. of creatine was used. All tissues were freed from food and blood, cut fine with scissors and added to the bottles. A small layer of toluene was used as a preservative in each bottle. These were shaken daily and kept in the incubator at 37°C. At the times indicated in the tables and chart the solutions were filtered and 5 cc. of the clear filtrate was added to a 50 cc. volumetric flask followed by the addition of 2 cc. of N HCl and 2 cc. of 5 per cent sodium tungstate. The whole was made up to the mark, shaken and filtered after standing 10 minutes. Ten cc. of the clear filtrate were added to a 100 cc. volumetric flask and autoclaved for 45 minutes with 2 cc. of N HCl. To the cooled flasks alkaline picrate was added. To another 100 cc. volumetric flask 10 cc. of the clear filtrate was added followed by the addition of alkaline picrate. After standing 10 minutes all flasks were made up to the mark with distilled water, shaken and the readings taken upon the Fisher electrophotometer. The difference between the total and preformed creatinine is expressed "creatine, as creatinine" in the tables and chart.

RESULTS AND DISCUSSION

When solutions of creatinine are incubated at 37°C. in the buffered solution some of the creatinine disappears (1). It is reasonable to believe that this creatinine is oxidized. Hence to all flasks in the first study oxygen gas was run in at the rate of about

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30 bubbles per minute for 1 day (table 1). Pitressin, in the presence of the different tissues, accelerated somewhat the oxidation of creatinine at the end of 1 days' incubation. At this time creatine formation from creatinine from highest to lowest by the various tissues was as follows: lung, kidney, liver, pancreas and muscle. At the end of

TABLE 1. CREATINE-CREATININE EQUILIBRIUM IN VARIOUS TYPES OF TISSUE INCUBATED WITH AND WITHOUT PITRESSIN

(Oxygen gas run in all flasks for 1 day)

Creatinine	Substances added to creatinine			After 1 day at 37°C.		After 2 days at 37°C.	
				Creatinine	Creatine, as Creatinine	Creatinine	Creatine as Creatinine
mg.		*cc.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*
50				22.0	1.8	3.0	2.1
50	Pitressin	1		16.3	1.2	0.0	4.5
50	Pitressin and spleen	1	1	15.5	5.3	0.0	6.0
	Pitressin, and spleen	1	1	0.0	0.0	0.0	0.0
	Spleen		1	0.0	0.0	0.0	0.0
50	Lung		1	9.3	**10.2**	0.0	**14.5**
50	Lung and pitressin	1	1	0.0	0.0	0.0	0.0
	Lung and pitressin	1	1	0.0	0.0	0.0	0.0
	Lung		1	0.0	0.0	0.0	0.0
50	Liver		1	6.0	6.5	0.0	**10.8**
50	Liver and pitressin	1	1	1.3	0.0	0.0	1.0
	Liver and pitressin	1	1	0.0	0.0	0.0	0.0
	Liver		1	0.0	0.0	0.0	0.0
50	Muscle		1	0.5	5.0	0.0	5.0
50	Muscle and pitressin	1	1	16.2	1.2	0.0	2.2
	Muscle and pitressin	1	1	0.0	1.0	0.0	0.1
	Muscle		1	0.0	1.1	0.1	0.2
50	Kidney		1	10.5	**10.0**	2.0	**16.0**
50	Kidney and pitressin	1	1	0.0	0.0	0.0	0.7
	Kidney and pitressin	1	1	0.0	0.0	0.0	1.2
	Kidney		1	0.0	0.0	0.0	0.9
50	Pancreas		1	6.0	6.5	0.0	4.0
50	Pancreas and pitressin	1	1	0.0	1.5	0.0	1.0
	Pancreas and pitressin	1	1	0.0	0.0	0.0	0.0
	Pancreas		1	0.0	0.0	0.0	0.0

the 2-day incubation period these relations were: kidney, lung, liver, muscle and pancreas. Hence fairly large increases in creatine formation from creatinine were possible in spite of the rapid oxidation of creatinine by oxygen.

In the second study no oxygen was admitted to the flasks (table 2). There was no effect on creatine formation at the end of 2 days except in the case of the stomach. At

TABLE 2. CREATINE-CREATININE EQUILIBRIUM IN INCUBATED TISSUES
(*Experiments without added oxygen*)

Creatinine	Substances added to creatinine in 1 gm. amounts	Days after Incubation at 37°C.							
		1		2		10		20	
		Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine
mg.		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
50		49.0	0.0	48.0	0.0	46.0	4.0	36.0	10.0
50	Spleen	46.0	0.0	46.0	0.0	40.0	5.0	36.0	11.0
	Spleen	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.5
50	Liver	46.0	0.0	45.0	0.0	37.0	9.0	32.0	13.0
	Liver	0.0	0.0	0.0	0.0	0.0	1.2	0.0	1.2
50	Lung	48.0	0.0	45.0	0.8	**26.0**	**19.0**	25.0	19.0
	Lung	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.9
50	Pancreas	46.5	0.0	46.8	0.0	40.2	7.0	36.0	9.5
	Pancreas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
50	Muscle	47.0	0.0	46.0	1.0	37.0	**13.0**	33.0	15.0
	Muscle	0.0	0.0	0.0	1.0	0.0	0.3	0.0	0.5
50	Kidney	48.0	0.0	47.0	0.0	38.0	6.0	33.0	12.0
	Kidney	0.0	0.0	0.0	0.0	0.0	0.8	0.0	1.1
50	Stomach	47.0	0.0	42.0	5.0	34.0	**13.0**	33.0	15.0
	Stomach	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
50	Muscle Liver Kidney	48.0	0.0	47.5	0.0	37.0	**11.0**	34.0	14.0
	Muscle[1] Liver[1] Kidney[1]	0.0	0.3	0.0	0.5	0.0	1.5	0.0	2.1

[1] These tissues were incubated in 50 cc. of the buffer solution.

the end of 10 days of incubation creatinine had disappeared faster in the presence of the tissues than in the control, lung being especially effective in this oxidation of creatinine. At the end of 20 days of incubation all values were practically the same, again with the exception of the lung tissue. Creatine formation from creatinine at the end of 10 days of incubation from highest to lowest was as follows: lung (muscle, stomach), liver, pancreas, kidney and spleen. These relations after 20 days' incubation were: lung (muscle, stomach), liver, kidney and spleen. The effect of lung tissue on the oxidation of creatinine in these studies was surprising. In other studies the addition of 0.15 M potassium cyanide, or boiling the tissues in water before addition to the bottles, blocked the oxidation and hydration of the creatinine.

Baker and Miller (2) observed that the concentrations of creatine and creatinine were roughly proportional in different tissues indicating an interrelationship of these two compounds in metabolism. The interesting observation was made that the concentration of creatinine in the spleen, lung, liver and pancreas was much lower than in an ultrafiltrate of plasma and this indicated that these tissues are concerned with the transformation of creatinine into creatine and other compounds.

TABLE 3. EFFECT OF THYROXIN ON CREATINE-CREATININE EQUILIBRIUM IN INCUBATED TISSUES

Creatinine	Substances added to creatinine	Days after Incubation at 37°C.													
		10		20		30		40		50		60		70	
		Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine
mg.		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
50		46	4	36	14	29	17	28	21	25	25	24	19	23	18
50	Thyroxin[1]	40	5	28	22	25	25	26	23	25	26	25	24	25	25
50	Spleen[2]	39	5	36	11	29	18	28	21	25	25	24	22	23	18
50	Thyroxin, and spleen	34	11	28	22	26	19	27	20	28	27	27	19	24	14
50	Thyroxin and liver	37	12	25	26	21	17	20	28	19	32	14	19	10	18
50	Liver	37	9	35	24	18	23	20	19	24	15	20	14	18	12
50	Thyroxin and kidney	37	12	33	15	29	16	30	16	30	24	28	17	28	11
50	Kidney	38	8	34	12	25	21	22	21	20	23	18	19	18	19
50	Thyroxin and muscle	33	12	31	19	28	22	29	22	28	28	12	22	10	20
50	Muscle	37	13	38	11	26	24	22	21	21	21	11	20	9	20
50	Thyroxin and intestine	39	9	36	14	34	15	17	22	15	10	10	11	7	9
50	Intestine	39	10	36	14	25	21	18	23	12	25	10	22	9	17
50	Muscle and liver and kidney	37	11	36	14	35	9	37	12	34	21	29	19	25	17
	Muscle[3] and liver[2] and kidney[3]	2	2	0	4	0	1	0	3	0	5	0	3	0	2
50	Lung	26	19	25	19	24	26	24	24	24	24	19	23	1,	25
50	Stomach	34	13	33	15	22	24	22	24	23	22	16	22	13	23

[1] Thyroxin, 1 mg.
[2] All tissues 2 gm.
[3] These tissues were incubated in 50 cc. of buffer solution.

From the above results it is obvious that most of the tissues studied contain enzymes which are capable of oxidizing and hydrating creatinine. The results of a longer period of incubation are recorded in table 3. The loss of creatinine was slightly larger with most tissues at 10 days' incubation than in the control. Then it was about the same as the control up to 50 days' incubation. From this time until 70 days' incubation creatinine was more rapidly lost in the presence of liver, kidney, muscle and intestine, than in the controls. The presence of thyroxin did not affect these results. Increased creatine formation over that in the control was noticed after 10 days of incubation in the presence of lung, kidney, liver, muscle, intestine and stomach. Lung again holds first place in the oxidation and hydration of creatinine at this time. Creatine formation at the end of 50 days of incubation was about the same in the control and experimental flasks. In the controls there was present 25 mg. of both creatine and creatinine at the end of 50 days of incubation.

The results of a shorter study with the posterior pituitary hormones and tissues are given in table 4. Pitressin and pitocin, with or without the tissues, had little effect on the creatinine-creatine equilibrium. This result was to be expected since these hormones do not act on administered creatinine in the rat but they do assist in the retention of body creatinine and its quantitative transformation into creatine (3). All tissues increase the rate of oxidation and hydration of creatinine. For some unknown reason the posterior pituitary hormones with lung or liver prevented somewhat the

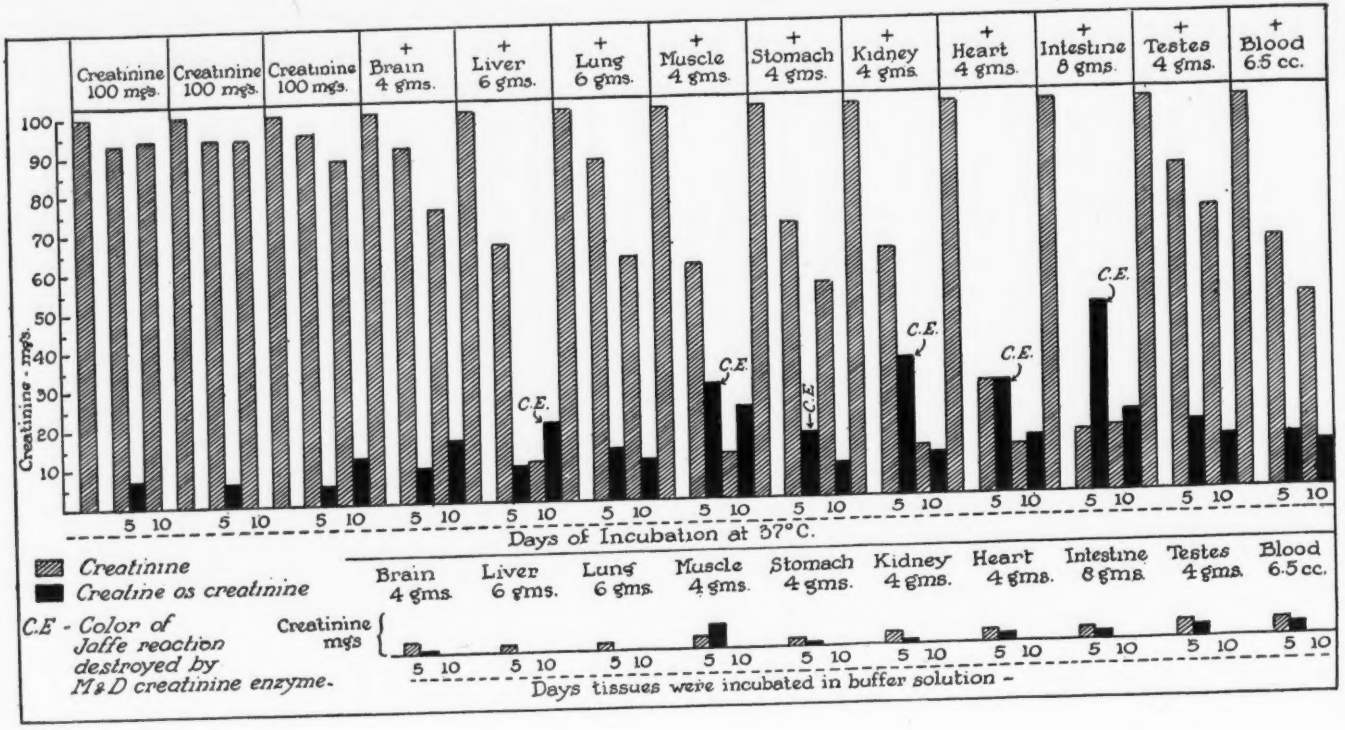
Creatinine 100 mgs.
Creatinine 100 mgs.
Creatinine 100 mgs.
+ Brain 4 gms.
+ Liver 6 gms.
+ Lung 6 gms.
+ Muscle 4 gms.
+ Stomach 4 gms.
+ Kidney 4 gms.
+ Heart 4 gms.
+ Intestine 8 gms.
+ Testes 4 gms.
+ Blood 6.5 cc.
Creatinine - mgs.
C.E.
Days of Incubation at 37°C.
Creatinine
Creatine as creatinine
C.E - Color of Jaffe reaction destroyed by M & D creatinine enzyme.
Brain 4 gms.
Liver 6 gms.
Lung 6 gms.
Muscle 4 gms.
Stomach 4 gms.
Kidney 4 gms.
Heart 4 gms.
Intestine 8 gms.
Testes 4 gms.
Blood 6.5 cc.
Creatinine mgs
Days tissues were incubated in buffer solution -

TABLE 4. EFFECT OF PITRESSIN AND PITOCIN ON CREATINE-CREATININE EQUILIBRIUM IN INCUBATED TISSUES

Creatinine	Substances added to creatinine[1]	Days after Incubation at 37°C.					
		5		10		15	
		Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine	Creatinine	Creatine, as Creatinine
mg.		mg.	mg.	mg.	mg.	mg.	mg.
50		47.0	1.0	46.0	4.5	40.0	7.8
50		48.0	0.0	46.0	1.0	40.0	5.0
50		50.1	0.0	49.0	0.0	48.0	0.0
50	Pitressin	46.3	4.0	43.2	6.5	43.2	7.2
50	Pitocin	48.1	2.0	41.6	6.9	39.5	9.3
50	Liver	46.0	3.0	38.2	9.0	30.6	**15.8**
50	Liver and pitressin	47.0	2.0	40.1	4.0	33.6	5.9
50	Liver and pitocin	47.0	2.0	39.2	4.3	34.3	6.5
50	Lung	47.5	0.0	42.0	3.9	20.0	**15.6**
50	Lung	41.6	2.6	35.0	5.0	23.0	**10.0**
50	Lung and pitressin	45.2	0.6	36.4	6.8	33.7	5.8
50	Lung and pitocin	44.2	1.5	41.7	3.6	35.6	6.3
50	Muscle	38.6	8.1	36.8	10.9	34.3	**12.1**
50	Muscle and pitressin	43.0	6.7	47.9	8.1	37.3	**11.1**
50	Muscle and pitocin	44.2	4.0	38.3	9.1	35.7	**13.3**
50	Kidney	**17.4**	7.8	9.1	**13.5**	5.2	**14.3**
50	Kidney and pitressin	25.4	0.0	19.0	4.0	7.5	**14.8**
50	Kidney and pitocin	23.9	5.2	19.2	7.7	8.7	**16.0**
50	Stomach	41.0	0.0	31.6	4.4	20.6	**11.0**
50	Intestine	31.5	**15.3**	30.4	9.1	27.6	8.0
50	Heart	31.5	4.5	23.0	7.0	17.7	**10.0**

[1] All hormones in 2 cc. amounts. All tissues in 2 gm. amounts.

hydration of creatinine to creatine. The rate of oxidation and hydration of creatinine from highest to lowest was as follows. *Oxidation*, kidney, heart, lung, stomach, intestine, liver and muscle; *hydration*, intestine (liver, lung, kidney, muscle, stomach and heart).

We suggest the following names for the above enzymes, *creatinine oxidase*, *creatinine hydrase*, and *creatine oxidase*. Since the oxidation and hydration of creatinine depend primarily upon the initial concentration of creatinine present in the solution, in the last study (fig. 1). 100 mg. of creatinine were used. The results from highest to lowest shown in the figure can be summarized as follows.

Creatinine oxidase (5 days)	heart, intestine, liver, blood, stomach, muscle, kidney (brain, lung, testes).
Creatinine oxidase (10 days)	kidney, heart, liver, muscle, intestine, blood, stomach, lung, testes and brain.
Creatinine hydrase (5 days)	intestine, kidney, muscle, heart, testes, stomach, blood, lung, liver and brain.
Creatinine oxidase (10 days)	intestine, kidney, heart, stomach, muscle, testes, lung, blood, brain, liver.

It is of interest in this connection that Bauman and Ingvaldsen (4) showed that creatine could be oxidized in vitro to methyl guanidine and oxalic acid. The color of the

TABLE 5. CREATINE-CREATININE EQUILIBRIUM IN MIXTURES OF TISSUES AND CREATINE.

Creatine	Gm. of Tissues added to creatine	Days after Incubation at 37°C.					
		5		10		20	
		Creatinine	Creatine as Creatinine	Creatinine	Creatine as Creatinine	Creatinine	Creatine as Creatinine
mg.		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
100		16.8	83.0	21.5	73.0	11.5	65.0
100		15.4	83.0	21.5	73.0	12.0	68.0
100		16.7	83.0	26.5	73.0	12.5	68.0
100	Liver, 6	19.5	80.0	17.5	64.0	10.5	65.0
	Liver, 6	2.0	0.2	0.0	0.0	0.0	0.0
100	Intestine, 8	19.5	83.0	12.5	64.0	10.5	65.0
	Intestine, 8	1.3	2.6	0.0	0.6	0.0	0.0
100	Muscle, 4	18.0	92.0	21.5	82.5	10.5	83.0
	Muscle, 4	3.0	6.0	1.0	2.2	0.0	0.0
100	Kidney, 4	18.0	89.0	16.5	73.0	10.5	72.0
	Kidney, 4	3.2	2.3	0.0	0.0	0.0	0.0
100	Lung, 4	16.8	85.0	15.0	72.0	10.7	72.0
	Lung, 4	2.0	1.0	0.0	0.0	0.0	0.0
100	Heart, 4	16.8	87.0	11.5	72.0	10.4	72.0
	Heart, 4	1.4	1.7	0.0	0.3	0.0	0.0
100	Testes, 4	16.0	88.2	19.0	73.0	10.5	76.0
	Testes, 4	1.6	3.0	0.0	1.1	0.0	0.0
100	Blood (7 cc.)	18.0	75.0	16.5	73.0	10.2	73.0
	Blood (7 cc.)	1.9	2.6	0.0	0.0	0.0	0.0
100	Stomach, 4	16.0	75.0	12.5	62.0	10.5	65.0
	Stomach, 4	2.0	1.5	0.0	0.0	0.0	0.0

Jaffe reaction in the total creatinine flasks was destroyed after incubation with the Miller and Dubos creatinine enzyme.

These results offer strong evidence for our views (5, 6, 7) that creatinine can be changed into creatine in situ in the tissues. They also show that the theory of Bloch and Schoenheimer (8) that creatinine cannot be changed into creatine in vivo is no longer tenable. We have discussed their results elsewhere (3). It would seem that the creatinuria of thyrotoxicosis or thyroid ingestion would be derived from the amino acids during metabolism or from free creatine of the tissues. Likewise the posterior pituitary hormones do not influence administered creatinine in the body but do cause a retention of body creatinine and assist in its quantitative transformation into creatine.

Gottlieb and Stangassinger (9) and Stangassinger (10) in 1907 stated that liver, muscle, spleen, lung and blood contained enzymes that destroyed both creatine and creatinine; they named these enzymes "creatase" and "creatinase" respectively, and stated that in these tissues there may occur a new formation of creatine from sources unknown. These results of Gottlieb and Stangassinger were confirmed by several other investigators. Hunter (11), in his monograph on creatine and creatinine, in discussing these experiments stated "those results in particular which implied an extensive destruction of creatine or creatinine during autolysis have been completely discredited. Creatase and creatinase have therefore now practically disappeared from the

literature." Our results recorded in this paper leave no doubt but that the different organs and tissues are able to cause an extensive loss of creatinine from solution and an increase in creatine formation.

After the above studies were completed it was decided to repeat the tests using 100 mg. of creatine instead of 100 mg. of creatinine. The results obtained are given in table 5 and may be summarized as follows.

a), 5 *days of incubation.* Creatinine formation from creatine, and creatine remaining in solution, was about the same in all flasks after correction for those amounts of creatine and creatinine formed and diffused out of the tissues themselves. There was no oxidation of creatine in the control solutions but there was some oxidation of creatine in the presence of blood and stomach.

b), 10 *days of incubation.* An average loss of 10 mg. of creatine occurred at this time accompanied by an average increase of 7 mg. of creatinine. In all tissues except muscle some creatinine was oxidized. Creatine was oxidized only in the presence of liver, intestine and stomach.

c), 20 *days of incubation.* There was an average oxidation of 6 mg. of creatine and 11 mg. of creatinine in the control flasks at this time, as compared to the amounts of these substances present in the flasks at the end of 10 days of incubation. At the end of 20 days of incubation the contents of creatine and creatinine in all flasks were about the same with the exception of muscle tissue.

The transformation of creatine into creatinine was no greater in the presence of the different tissues than it was in the control flasks. It is, therefore, evident that no enzyme occurs in these tissues which is capable of causing this change. We do not deny the possibility of this transformation but we have yet to observe it in any of our studies (3, 5, 6, 12–15).

The disappearance or destruction of creatine and creatinine when they are ingested would, in the light of the results of the present studies, possibly be due to their oxidation into other substances, creatinine into sarcosine (16) and creatine into methyl guanidine and oxalic acid (4).

SUMMARY AND CONCLUSIONS

The effect of incubating different tissues with and without thyroxin, pitressin and pitocin, upon creatine-creatinine equilibrium in vitro was studied. The presence of 3 enzymes in all tissues studied was shown. Their effect on creatinine and creatine oxidation and on creatinine hydration from highest to lowest in the different tissues was as follows.

Creatinine oxidase, heart, intestine, kidney and lung.

Creatinine hydrase, intestine, kidney, muscle and heart.

Creatine oxidase, intestine, kidney, heart and stomach.

These enzymes were also present in smaller amounts in the other tissues studied.

Boiling the tissues in water, or the addition of 0.15 M KCN, blocked the action of these enzymes.

Incubation of the solutions in the total creatinine flasks in the last creatinine study with the specific creatinine enzyme of Miller and Dubos caused a disappearance of the color of the Jaffe reaction after the addition of alkaline picrate.

Thyroxin, pitressin and pitocin had little effect upon the above reactions. The effects of these hormones upon creatine-creatinine metabolism in vivo was briefly discussed.

No evidence was obtained that creatine, incubated in the same way with the tissues as creatinine, was transformed into creatinine. The existence of the enzyme creatine anhydrase in the tissues studied, does not occur.

We wish to thank Dr. Oliver Kamm of Parke, Davis & Company for the samples of pitressin and pitocin used in these studies.

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CHANGES IN THE FAT PERCENTAGE AND FAT YIELD OF DAIRY COWS WITH INJECTIONS OF AN ANTERIOR PITUITARY PREPARATION[1]

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SEVERAL INVESTIGATORS have shown that certain extracts from the anterior pituitary have marked effects on the fat metabolism of various laboratory animals (1–5). The present report concerns the effect of anterior pituitary extracts on the fat production of dairy cattle.

EXPERIMENTAL PROCEDURE

The anterior pituitary extract used in this study was the alkaline extract described by Best and Campbell (3) and was prepared in this laboratory according to the method outlined in that report. No quantitative assay was made on the preparation. Tests were made, however, to demonstrate the liver-fat activity of the extracted material. The preparation was injected for 5 day-periods in 500 mg. amounts. For purposes of comparison a prolactin preparation prepared in this laboratory by the method of Bates and Riddle (6) was given in 500 mg. amounts to the same 4 cows during another 5-day period. The prolactin used assayed approximately 2 units per mg. by the method of McShan and Turner (7). The prolactin was also tested for liver-fat activity. All injections were given subcutaneously.

Four cows in declining lactation were used for experimentation. Three of these cows were of the Holstein breed and one (*cow* 3) was a Jersey. Daily records of milk production were obtained. The fat percentage of the milk was determined on 3-day composite samples and the daily fat production calculated from the data so obtained.

RESULTS

The comparative liver-fat activity of the fat metabolism preparation and the prolactin is shown in table 1. Guinea pigs approximately 250 gm. in weight were used for the liver-fat determinations. Liver fat was determined by the saponification procedure of Leathes and Raper (8).

TABLE 1. COMPARATIVE LIVER-FAT ACTIVITY OF THE FAT METABOLISM PREPARATION AND PROLACTIN

Preparation	Liver fat, av.	Amount injected per animal	No. of animals
	%	mg.	
Fat metabolism preparation	10.2	30	16
Prolactin	6.3	75	16
Controls	5.7		14

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The effect of the fat metabolism preparation on milk volume, percentage of fat and fat production is shown in table 2. The results for milk volume and fat production are expressed in pounds.

TABLE 2. EFFECT OF THE FAT METABOLISM PREPARATION ON MILK VOLUME, FAT PERCENTAGE AND FAT PRODUCTION

Cow 1			Cow 2			Cow 3			Cow 4		
Milk vol. lb.	% fat	Fat prod. lb.	Milk vol. lb.	% fat	Fat prod. lb.	Milk vol. lb.	% fat	Fat prod. lb.	Milk vol. lb.	% fat	Fat prod. lb.
Pre-injection period											
16.6		.47	15.6		.56	10.7		.58	14.9		.52
17.1		.46	16.2		.58	11.2		.62	14.7		.51
16.8	2.7	.45	16.3	3.6	.59	11.8	5.4	.64	15.2	3.5	.53
15.6		.41	15.7		.52	11.0		.53	13.8		.50
16.4		.43	16.4		.54	10.4		.50	14.0		.50
17.3	2.6	.45	15.6	3.3	.51	11.1	4.8	.53	15.4	3.6	.55
13.8		.41	15.6		.56	10.2		.53	14.8		.52
16.1		.48	16.0		.58	11.4		.59	16.1		.56
16.4	3.0	.49	15.5	3.6	.56	11.3	5.2	.59	14.3	3.5	.50
Injection period											
14.8		.44	16.9		.61	11.7		.70	13.6		.54
17.3		.52	17.1		.61	13.8		.83	16.1		.64
18.4	3.0	.55	19.2	3.6	.69	14.8	6.0	.89	16.1	4.0	.64
17.8		.52	16.8		.75	14.9		1.09	14.6		.71
16.3		.47	16.4		.74	15.2		1.11	13.9		.68
Post-injection period											
14.5	2.9	.42	15.6	4.5	.70	16.0	7.3	1.17	15.6	4.9	.76
14.2		.41	15.3		.61	14.8		.96	14.1		.66
15.0		.43	16.3		.65	14.0		.91	13.5		.63
15.6	2.9	.45	16.2	4.0	.65	13.0	6.5	.84	13.6	4.7	.64
15.0		.42	16.3		.62	12.2		.63	13.7		.62
15.4		.43	15.6		.59	10.6		.55	12.6		.57
14.4	2.8	.40	17.6	3.8	.67	9.4	5.2	.49	12.9	4.5	.58
15.0		.46	14.3		.53	11.9		.54	13.2		.53
14.4		.45	15.5		.57	11.0		.49	12.1		.48
13.6	3.1	.42	14.7	3.7	.55	11.3	4.5	.51	12.7	4.0	.51

When prolactin was injected into these same 4 cows for a similar period the results recorded in table 3 were obtained.

DISCUSSION OF RESULTS

The data indicate that the fat metabolism preparation used in this study caused a marked increase in the fat production in 3 of the 4 cows tested. The reason for the failure of the 4th cow to respond to this preparation is not clear. In one instance (*cow* 3) this increase was nearly 100% greater than the fat production for any one day in the period preceding the injections. The other 2 cows (*cows* 2 and 4) showed increases amounting to nearly 50% over that of the pre-injection period.

The increased fat production in these 3 animals was mainly due to the marked rise in the percentage of fat in the milk. In spite of the fact that increases in milk volume occurred in every instance, a change which is normally accompanied by a decrease in the percentage of fat, the fat percentage of the milk in these animals increased

remarkably (table 2). Similar increases in milk volume which resulted when prolactin was injected (table 3) had only very slight effect on the daily fat production and no detectable effect on the fat percentage in the milk. These latter results agree with those obtained by Folley and Young (9). The increases in milk volume noted following injection of the fat metabolism preparation were probably due to the slight prolactin activity which this preparation possesses (10).

The marked increases in both fat production and percentage of fat in the milk

TABLE 3. EFFECT OF PROLACTIN ON MILK VOLUME, FAT PERCENTAGE AND FAT PRODUCTION

Cow 1			Cow 2			Cow 3			Cow 4		
Milk vol. lb.	% fat	Fat prod. lb.	Milk vol. lb.	% fat	Fat prod. lb.	Milk vol. lb.	% fat	Fat prod. lb.	Milk vol. lb.	% fat	Fat prod. lb.
Pre-injection period											
17.9		.55	14.0		.56	12.0		.66	12.1		.43
17.8	3.1	.55	16.3	4.0	.65	12.4		.68	12.4		.45
17.0		.51	14.6		.54	12.0	5.5	.66	11.7	3.6	.42
17.9		.54	15.5		.57	11.7		.66	11.5		.43
18.0	3.0	.54	16.0	3.7	.59	12.3		.69	10.5		.39
16.7		.48	15.2		.59	12.5	5.6	.70	10.6	3.7	.39
16.7		.48	14.5		.57	12.9		.71	11.8		.45
18.0	2.9	.52	14.9	3.9	.58	12.3		.68	10.8		.41
17.5		.47	16.9		.61	11.6	5.5	.64	11.6	3.8	.44
Injection period											
19.7		.53	16.9		.61	13.1		.69	13.0		.47
20.3	2.7	.55	17.0	3.6	.61	12.0		.64	13.5		.49
20.0		.52	16.7		.57	12.1	5.3	.64	13.5	3.6	.49
19.5		.51	17.3		.59	11.5		.63	14.0		.55
18.0	2.6	.47	16.4	3.4	.56	11.0		.61	13.5		.53
Post-injection period											
18.4		.50	16.5		.61	11.8	5.5	.65	13.6	3.9	.53
19.6		.53	18.2		.67	12.2		.67	14.2		.54
18.3	2.7	.49	16.5	3.7	.61	11.1		.61	13.5		.51
18.1		.54	16.4		.62	10.4	5.5	.57	14.3	3.8	.54
18.5		.56	16.8		.64	10.9		.52	13.4		.51
17.8	3.0	.54	15.9	3.8	.60	11.6		.56	13.4		.51
18.9		.55	15.7		.60	10.9	4.8	.52	13.5	3.8	.51
17.2		.50	15.3		.58	10.8		.61	12.5		.49
17.7	2.9	.51	15.7	3.8	.60	11.1		.63	14.0		.55
						10.0	5.7	.57	13.2	3.9	.51

which resulted from injections of this fat metabolism preparation, are similar to the increases produced by desiccated thyroid or thyroxin (11, 12) and it might be argued that contamination of the preparation with thyrotropic hormone could account for the changes produced. However, Folley and Young (9) have shown that thyrotropic preparations prepared in their laboratory have little effect on total fat production and no detectable effect on the percentage of fat of the milk. It therefore seems probable that the effects produced in these trials are the result of a substance which in addition to its effect on liver fat and ketone production affects fat metabolism of the mammary gland; at least they are not effects due to thyrotropic contamination of the preparation used.

These findings are also additional evidence that the secretion of milk is not the re-

sult of the action of a single substance, the lactogenic hormone. This view has been expressed previously particularly by Folley and Young (9) and Azimov and his associates (13). These investigators have shown that the effect of prolactin on milk secretion is enhanced when other anterior pituitary hormones are present in the prolactin preparation. The fat metabolism preparation used in the present investigation primarily affected the amount of fat secreted into the milk by the mammary gland and these results, when considered in the light of previous work would suggest that each component of the secretory process may be controlled by a separate specific hormone and that lactation is the result of the combined activity of these various substances.

SUMMARY

An anterior pituitary preparation possessing marked liver-fat activity has been shown to affect fat production of the mammary gland of 3 dairy cows. This preparation caused marked increases in both the total fat produced and in the percentage of fat of the milk.

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PROTECTIVE ACTION OF CARDIAC GLYCOSIDES AND ADRENAL CORTICAL HORMONE AGAINST THYROXIN[1]

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THE POSSIBILITY of a relationship between the physiologic activity of the cardiac steroid-glycosides and those derived from the adrenal cortex was suggested by Zwemer, Lowenstein, and Pines (1). They demonstrated the ability of certain of these glycosides to protect animals against insulin shock and also confirmed a cortical-like effect of strophanthin upon plasma potassium and blood sugar levels previously reported by Ginsburg (2).

On the basis of clinical and experimental findings, a definite degree of antagonism would seem to exist between the thyroid and adrenal cortical hormones (3–15). Clinically, the hyperthyroid patient presents many of the accepted criteria for the administration of drugs of the digitalis series: tachycardia, and varying degrees of failure, with or without fibrillation. We therefore felt that an investigation of possible cortical-like effects of cardiac steroids, in relation to experimentally induced hyperthyroidism, would be in order.

MATERIALS AND METHODS

Sixty male guinea pigs and 250 male mice of the Rockland Swiss strain were used in this work. Guinea pigs varying from 160 to 800 gm. in weight were used in order that any differential effects referable to maturity or immaturity, if present, might be observed. Approximately equal weight distribution was maintained in control and experimental groups. Results are reported for representative two-week treatment periods in each group of animals.

The materials used were as follows:

Thyroxin,[2] 1:1000 solution prepared at frequent intervals to insure potency
A water soluble extract of the whole thyroid gland, standardized on the basis of organic and inorganic iodine present[2]
A mixture of the crystalline lanatosides A, B and C[3]
The crystalline steroid-glycoside of Strophanthus-Kombe[3]
Adrenal cortical extract[4]
Desoxycorticosterone acetate[2]

All medication was given daily by subcutaneous or intraperitoneal injection. The diet was constant for the control and experimental groups. Representative animals

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[2] The thyroxin, thyroid extract (Thyranon) and desoxycorticosterone acetate were supplied by Roche-Organon, Inc., Nutley, New Jersey.

[3] The mixture of lanatosides A, B, and C (Digilanid) and the crystalline steroid-glycoside (K-Strophanthoside) were supplied by Sandoz, Inc., New York City.

[4] The adrenal cortical extract (Cortin) was supplied by the Upjohn Company, Kalamazoo, Michigan.

TABLE 1, A AND B. COMPARATIVE WEIGHT CHANGES IN MICE OVER TWO-WEEK PERIODS

Group	Daily Treatment		Initial Wt.	Final Wt.	Wt. Change
	Material	Dosage			
		μg./gm.	gm.	gm.	%
1A	Lanatosides A, B, C	2	24.6	23.3	− 5.3
2A		4	24.0	22.5	− 6.2
3A		6	23.0	19.7	−14.3
1C	Synthetic thyroxin	10	29.8	30.9	+3.7
2C		25	27.9	27.7	−0.7
3C		50	23.5	23.8	+1.0
EC	Thyroid extract	.01 cc./gm.	26.4	26.3	−0.4
4C[1]	Inj. saline		21.5	22.4	+4.0
5C[1]	No treatment		22.0	23.1	+5.0

[1] Controls.

All dosages of lanatosides shown above are apparently toxic. Synthetic thyroxin is apparently incapable of inducing consistent weight loss in young healthy mature mice. The same may be said for whole thyroid hormone in the dosage used.

Group	Daily Treatment	Initial Wt.	Final Wt.	Wt. Change
		gm.	gm.	%
FC	0.01 cc./gm. thyroid ext. 0.1 μg./gm. strophanthin	15.3	19.9	+30.1
GC	0.01 cc./gm. thyroid ext.	15.0	20.4	+36.0
HC	Controls	15.0	19.0	+26.7
AD1	Unilateral adrenalec. 0.01 cc./gm. thyroid ext.	27.0	24.5	−9.2
AD2	Unilateral adrenalec. 0.01 cc./gm. thyroid ext. 0.04 μg./gm. strophanthin	25.7	26.8	+4.3
BD	Unilateral adrenalec. controls	21.7	22.9	+5.5

Note *groups FC, GC,* and *HC.* The inhibition by strophanthin of the weight gain induced by thyroid hormone in immature mice, may be a toxic or therapeutic effect.

The gain in weight in *group AD* apparently represents a definite inhibition of katabolism.

were killed at different stages of the experimental work in order that thyroid and suprarenal tissue might be obtained for histologic examination and, in addition, that any gross pathology might be observed.

EXPERIMENTAL RESULTS

Weight changes. Weight changes over representative 2-week periods were recorded: *a*), in groups of mature mice receiving 0.1, 0.2 and 0.3 lethal doses of the mixed crystalline lanatosides, respectively (M.L.D. 20 micrograms per gm.); *b*), in groups of mature mice receiving 10, 25, and 50 micrograms (μg.) of thyroxin per gm., respectively; *c*), in a group of mature mice receiving 0.01 cc. per gm. of whole thyroid extract; and *d*), in control groups receiving either no treatment or saline injections daily (table 1A). All of the mice receiving the crystalline lanatosides[2] lost weight, roughly in proportion to the dosage. None of the thyroxin-treated mice lost weight. We were, therefore, forced to conclude that the normal adult mouse was unsuited for this type of investigation.

TABLE 2. COMPARATIVE WEIGHT CHANGES IN GUINEA PIGS OVER TWO-WEEK PERIODS

Animal Number	Original Weight gm.	Weight Change %
Group 1, controls		
6A	840	+4.4
7A	623	+4.8
8A	607	+7.5
9	399	+26.0
5A	375	+21.8
12	312	+40.3
11	286	+29.0
5	285	+31.5
Group 2, synthetic thyroxin, 1 μg./gm.		
22	592	−34.1
24	585	−25.6
21	454	−19.2
15	390	−17.4
13	345	−17.3
14	337	−19.8
23	285	−15.7
16	255	−11.7
Group 3, thyroid, 1 μg./gm.; digitalis, 0.2 μg./gm.		
21	352	+3.7
15	308	+12.0
23	255	+11.8
16	233	0
Group 4, digitalis 0.2 μg./gm.; thyroid 1 μg./gm.		
A 4	742	−31.2
A 2	675	−18.9
A 3	652	−26.3
A 1	480	−13.1
B 18	349	−6.8
B 19	334	−7.4
B 20	276	−6.5
B 17	253	+7.9
Group 5, cortical extract; thyroid 1 μg./gm.		
29	268 +2.	+11.9
30	252	+6.3
32	219	−1.8
31	189	+11.6
Group 6, desoxycorticosterone, 4 μg./gm.; thyroid, 1 μg./gm.		
25	465	−0.6
27	286	+2.4
28	226	+11.5

NOTE—*Animals No. 1, 2, 3,* and *4* had received preliminary treatment with lanatosides A B and C for several weeks prior to the beginning of thyroxin administration. These weight changes, therefore, probably represent an accentuation of the katabolic effect of thyroxin by an amount of digitalis which is toxic at this metabolic level. *Animals No. 17–20,* inclusive, had received the same substance for a shorter period of time. These, together with *animals No. 15, 16, 21* and *23,* apparently represent an inhibition of katabolism by doses of digitalis which are therapeutic at this metabolic level.

Two-tenths μg./gm. of the mixed lanatosides A, B and C is approximately 0.2 lethal. Relative excretion-accumulation is unknown.

Three groups of immature male mice were selected (table 1B), one of which received thyroid hormone;[2] another was given the same dosage of thyroid hormone, plus 1 microgram per gm. strophanthin; and the third was weighed daily but received no treatment. Those receiving the thyroid extract gained weight much more rapidly than the controls. This apparently indicated a stimulation of anabolism. Those receiving thyroid and strophanthin had a two-week percentage weight gain midway between that of the controls and those receiving thyroid extract. This may represent an inhibition of the metabolic stimulation.

A group of unilaterally adrenalectomized mature male mice was treated with thyroid hormone for 2 weeks. Moderate weight loss occurred. This weight loss was practically obliterated within 4 days after the animals received 0.04 micrograms per gm. of the steroid-glycoside[3] daily, together with the thyroid hormone (table 1B).

The results obtained in the guinea pigs are shown in tables 2 and 3. Summarized, they are essentially as follows.

Adrenal cortical extract,[4] in dosage of 2 cc. per kg., has a definitely protective effect against the weight loss induced by fairly large dosage of thyroxin (1 μg. per gm.). This protection ceased after continued administration of thyroxin. The period of protection in our animals was approximately 2 weeks (table 2, *group 5*).

Desoxycorticosterone acetate, in dosage of 4 micrograms per gm., exerted a sig-

TABLE 3. COMPARATIVE WEIGHT CHANGES IN THYROXIN-STROPHANTHIN-TREATED GUINEA PIGS OVER TWO-WEEK PERIODS

Group 7			Group 8		
Thyroxin 0.25 μg./gm.			Thyroxin 0.25 μ/gm. Steroid Glycoside of Strophanthus 0.0125–0.025 μg./gm.		
Animal number	Original weight	Weight change	Animal number	Original weight	Weight change
	gm.	%		gm.	%
42	258	+10.0	54	265	+3.4
44	240	+10.4	56	228	+8.7
45	227	− 5.7	57	227	+2.6
47	206	+14.5	59	199	+9.0
43	196	−11.7	62	188	+4.3
46	172	+12.2	61	186	+6.0
			58	176	+5.7
			60	171	+1.0

Average weight gain for normal animals in this weight range, varies between 30 and 40%.
M.L.D. of steroid glycoside strophanthus for guinea pigs is 0.25 μg./gm.
Dosage of 0.025 μg./gm. (1/10 M.L.D.) was given during first part of 2-week period; consistent weight losses occurred. After dosage of 1/20 M.L.D. consistent gain ensued; 1/10 M.L.D. apparently enhances katabolic effect of 0.25 μg./gm. synthetic thyroxin. 1/20 M.L.D. of strophanthin tends to lessen katabolic action of thyroxin.

nificant but lesser degree of protection against thyroxin than did natural adrenal cortical extract (table 2, *group 6*).

Guinea pigs receiving prolonged preliminary digitalis administration (0.2 μg. per gm., daily), when given thyroxin in addition to the digitalis, were more toxic and lost weight with equal or greater rapidity than animals receiving only an equal dose of thyroxin (table 2, *group 4A*).

Guinea pigs receiving the same dosage of digitalis for 2 to 6 days prior to the beginning of thyroxin administration, either actually gained or lost at a significantly slower rate than those receiving thyroxin alone (table 2, *group 4B*).

Animals which had experienced extreme and maintained weight loss from long-continued thyroxin administration, either gained or stopped losing when digitalis was administered (table 2, *group 3*).

The results shown in table 3, on first inspection, seem inconclusive inasmuch as the average percentage weight change in animals receiving 0.25 μg. per gm. of thyroxin and those receiving this medication plus simultaneous strophanthin is essentially the same. The animals in the latter group, however, received 0.1 the lethal dose of strophanthin during the first 9 days and 0.05 lethal dose strophanthin over the last 5 days. Most of the weight gain occurred during this time. In other words, 0.025 μg. per gm. of steroid glycoside[3] administered daily apparently accentuates the katabolic effect of 0.25 μg. per gm. of thyroxin. Half of this dose of strophanthin seems to inhibit the stimulation of katabolism resulting from 0.25 μg. per gm. of thyroxin.

Tissue changes. In the guinea pig, the most constant gross variable was the relative amount of perirenal fat present. Thyroxin-treated animals had slight, marked, or complete loss of perirenal fat in proportion to the intensity of thyroid hormone administration. Animals receiving only digitalis or strophanthin showed no essential variation from normal. Those receiving thyroid hormone plus cardiac steroid-glycosides did not differ in marked degree in this respect from animals receiving only thyroid hormone, even in cases where a positive weight balance occurred. This would suggest that the weight differential was due to lessened muscle breakdown or to tissue fluid accumu-

lation. No gross evidence of edema existed; if anything, this latter group seemed somewhat dehydrated; hence, the former interpretation is probably correct.

Histologic changes in the suprarenal glands of both mice and guinea pigs receiving cardiac glycosides in therapeutic dosage were essentially the same as those described by Zwemer et al. (1). Animals receiving toxic doses, either as a single lethal dose or in the form of prolonged treatment with digitalis, in many instances showed hemorrhagic involvement of the reticular and fascicular zones of the cortex. Changes varying in degree from those indicative of mild stimulation to exhaustion were observed in the glands of the animals receiving only thyroxin. These changes were directly proportional to the dosage and duration of administration.

The adrenals of animals receiving thyroxin plus adrenal cortical extract showed evidence of a gland functioning under fairly acute demand. The capsular and glomerular zones were somewhat thicker than normal. The fascicular zone showed a decreased ratio of cytoplasm to nuclear material.

The adrenal cortex of animals receiving thyroxin plus cardiac glycosides showed all stages of activity. Those animals which had lost weight rapidly from the combined effects of thyroxin and digitalis, showed hemorrhagic changes and loss of zonal differentiation. Those receiving small dosages of digitalis plus thyroxin had a cortical picture very similar to that observed in the cortin-thyroxin-treated animals. Hematoxylin and eosin was the only staining procedure used in this series, hence a true evaluation of lipoid production and storage could not be made.

We have so far been unable to establish any common denominator for histologic changes in the thyroid glands, either on the basis of staining properties, cell height, or colloid accumulation. Marked but inconstant variations were observed in the glands of some of the thyroxin-cortin and thyroxin-digitalis-treated animals.

DISCUSSION

These findings would suggest that a relatively small dosage of cardiac steroid-glycosides exerts a protective effect against at least some part of the metabolic stimulation produced by repeated doses of thyroxin, in a manner similar to that exerted by the hormone of the adrenal cortex. It also indicates a lack of such protection, or even an accentuation of the toxic effect of thyroxin, by larger doses of cardiac glycosides, doses which, when administered to normal animals, are in themselves nontoxic. Despite the repeated demonstration of the antagonistic effects of cortical extract and thyroxin (3–15) no satisfactory mechanism has been established to explain such a relationship. A brief consideration of certain known physiologic effects of thyroxin, synthetic and natural cortical hormones, and cardiac glycosides, relative to potassium, may help to clarify this subject.

Brewer (16) has demonstrated an elevation of plasma potassium, with elevations in metabolic rate. The work of Schachter and Huntington (24) tends to confirm this observation and suggests that administration of thyroid hormone produces a definite rise in plasma potassium.

The ability of therapeutic dosage of adrenal cortical hormone to lower the plasma potassium level has been adequately demonstrated (17). Similar effects of strophanthin have been previously referred to (1–2).

Ferrebee et al. (18) have recently shown that large (toxic?) doses of desoxycorticosterone acetate produce a reversal of this process, namely, a loss of potassium from cells, with replacement by sodium. It has been shown similarly that toxic doses of digitalis produce a potassium loss from muscle cells (19–22).

From the clinical side, Christian and Swift, in discussing a paper by Plummer (23) emphasize that factors which increase the metabolic rate lessen the tolerance for

digitalis, and Plummer in the same paper refers to the dangers of cerebral edema when digitalis is administered to hyperthyroid patients (replacement of K by Na in brain cells?).

There is reason to believe that the antagonistic effects of thyroxin-adrenal cortical hormone, and thyroxin-cardiac glycosides, are at least in part based upon the effects of all those substances upon potassium. We advance the following reasons for this hypothesis.

a) Factors which increase the metabolic rate cause a loss of intracellular potassium and, consequently, an elevation of the plasma potassium level, essentially a phenomenon of katabolism. *b*). The suprarenal cortical hormone produces a lowering of plasma potassium, presumably by restoring a normal balance between intracellular and extracellular potassium. *c*). Cardiac glycosides in therapeutic range have an effect upon potassium similar to that of adrenal cortical hormone. The therapeutic level, however, varies indirectly with the metabolic rate. *d*). Toxic doses of cardiac glycosides have an opposite effect upon intracellular potassium from therapeutic doses, in a manner similar to that demonstrated for desoxycorticosterone acetate.

Work now in progress in this laboratory will attempt to further determine the soundness of this hypothesis.

SUMMARY

Therapeutic dosage of cardiac steroid-glycosides prevents a weight loss in thyroxin-treated animals similar to that obtained with adrenal cortical hormone and desoxycorticosterone acetate. Larger dosage of these cardiac glycosides either fails to modify such weight loss or actually increases it.

Histologic changes in the suprarenal cortex of animals receiving therapeutic dosage of cardiac glycosides and thyroxin, are similar to those observed in animals treated with cortin and thyroxin.

We wish to express our thanks to Dr. Thomas Snyder and his assistants for their aid in the histologic work.

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SOME EFFECTS OF THYROTROPIC HORMONE ON THE REPRODUCTIVE SYSTEM OF NORMAL, THYROIDECTOMIZED OR ADRENALECTOMIZED FEMALE RATS

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A RELATIONSHIP BETWEEN the hyperthyroid state and the female reproductive system is fairly well established. Hyperthyroidism, produced by injection of thyroxin or feeding desiccated thyroid, may cause anestrus, pseudopregnancy, resorption or prolonged gestation in the rat (1, 2, 3). The effect of thyrotropic hormone on the reproductive system of the rat has not been extensively investigated because rats have been shown to be relatively unresponsive to this hormone. Sufficiently large dosage, however, will increase metabolism and produce other indications of hyperthyroidism (4). The guinea pig, on the other hand, is very sensitive to thyrotropic hormone, showing not only stimulation of the thyroid but also luteinization of the ovaries (5, 6, 7).

The following experiments were undertaken to determine: *a*) whether the ovaries of immature and mature rats would become luteinized after injections of thyrotropic hormone; and if so, whether the luteinization was due to a hyperthyroid condition or to gonadotropic substances in the thyrotropic preparation; and *b*) whether such luteinized ovaries would help prolong the survival time of adrenalectomized rats[1] or prepare the uterus for the production of traumatic placentomata.

MATERIAL AND METHODS

Immature and mature female albino rats (inbred Wistar strain) averaging 82 and 125 gm. in weight respectively, were reared in a thermostatically controlled room on a diet of commercial dog biscuit and tap water ad libitum. Daily weight records and vaginal smears were made for 2 weeks before starting injections, adrenalectomizing or thyroidectomizing the animals, and continued throughout the experimental period. Tissues taken from the control or thyroidectomized animals at autopsy, and from the adrenalectomized rats at the time of death, or shortly thereafter, were fixed in Bouin's solution. Serial sections of one ovary and one lobe of the thyroid, and sample sections of the uteri were stained with hematoxylin and eosin for histological study.

RESULTS

Normal Animals Treated with Thyrotropic Hormone

A. *Mature females.* Nineteen females were given daily intraperitoneal injections of thyrotropic hormone[2] in the dosage and for the periods indicated in table 1. His-

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[1] It has been found that luteinization of the rat's ovaries by gonadotropic hormones, the presence of the corpora lutea of pregnancy and lactation, or the injection of progesterone will prolong the survival time of adrenalectomized rats (8–15).

[2] The thyrotropin (Antuitrin T) was generously provided by Parke, Davis and Company, Detroit,

tologically the thyroids showed high columnar epithelium in the follicles, with liquefaction and contraction of the colloid (fig. 1 and 2). Regardless of the stage of the cycle at the time of the first injection, the animals subsequently came into diestrus. These periods of diestrus varied from 2 to 15 days and in some instances cycles were resumed in spite of the continued injections.[3]

In addition to the normal complement of variously aged corpora lutea as well as developing and atretic follicles, the ovaries of all the treated animals contained unruptured follicles in the process of luteinization. These luteinized follicles shall be referred to as pseudo-lutein bodies. Not all these later structures were in the same stage of development in any one ovary. The various stages were graded as: *stage 1*, in which a small amount of lutein tissue was apparently developed from the mural granulosa of a mature follicle containing a normal-appearing ovum (fig. 3 and 4); *stage 2*, in which a small antrum containing follicular fluid and a single or divided ovum was present in the follicle and most of the granulosa, except for the cumulus, was replaced by lutein tissue (fig. 5); *stage 3*, in which the antrum was obliterated, a little granulosa tissue remaining around a single or divided ovum, and luteal tissue was well formed (fig. 6). Intrafollicular hemorrhage occurred in the formation of many pseudo-lutein bodies. In all three types the theca interna and externa were present.

There appeared to be no significant correlation between the size and duration of dosage, the phase of the cycle at the time of the first injection, and the number of various stages of pseudo-lutein bodies formed. If an interval of 20 to 24 days elapsed between the initial 5 to 6 day period of injection and resumption of treatment, pseudo-lutein bodies could still be produced in the ovaries (table 1, *rat 18* and *19*), indicating that any unresponsiveness produced by the first series of injections was not effective after this period.

B. Immature females. Four females, 23 days of age, were injected once daily with 0.1 or 0.2 cc. of thyrotropic hormone over a period of 5 days and killed on the 6th day. As compared with littermate controls, this treatment caused no suppression of growth or change in ovarian weight.[4] Histologically the thyroids showed evidence of stimulation and the ovaries contained from 15 to 23 pseudo-lutein bodies.

Although unruptured follicles may become luteinized in the normal ovary (18, 19), the number of pseudo-lutein bodies observed in the present studies after injections of thyrotropic hormone, would indicate that this was more than a chance finding, for only occasionally do ovaries of control animals of similar age contain 1 or 2 luteinized follicles.

Thyroidectomized Animals With or Without Thyrotropic Hormone Treatment

In order to determine whether the pseudo-lutein body formation was due to a state of hyperthyroidism or to gonadotropic hormones in the thyrotropic preparation, the following studies were made on thyroidectomized animals. Twelve mature females were thyroidectomized. Five to 6 successive daily injections of 0.2 cc. of thyrotropic hormone were begun 1 to 4 days postoperatively in 4 rats, and 22 and 24 days postoperatively in 2 others; the remaining 6 serving as controls. The latter, after remaining in anestrus 4 to 15 days after operation, showed normal cycles. The treated rats showed a similar estrous picture except for the anestrus occurring during the periods of injection.

Mich., and the Thyrotrophic Factor by Ayerst, McKenna and Harrison, Rouses Point, N. Y. Both preparations were assayed to contain 50 to 100 guinea pig units per cc.

[3] It has been reported that guinea pigs and rats may become unresponsive after comparative treatment with thyrotropic hormone (5, 6, 16, 17).

[4] Stephens and Allen (7) also found no change in the weight of the ovaries from 25-day-old rats treated with 0.6 cc. of this hormone given over a period of 3 days.

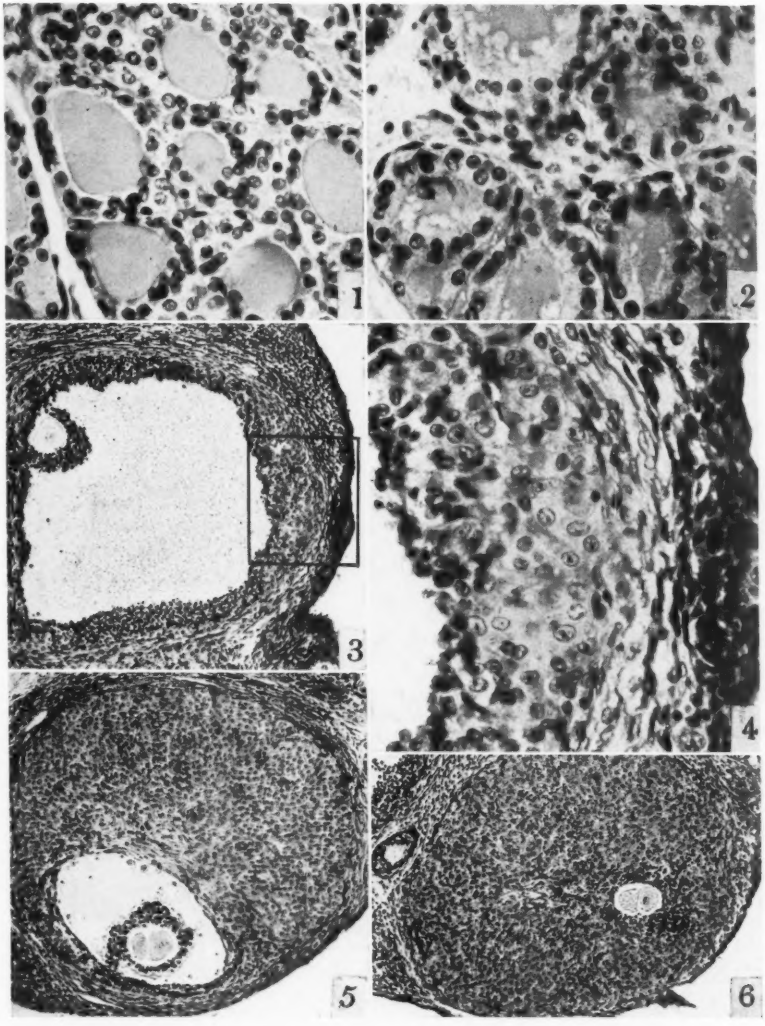

Fig. 1. SECTION OF THYROID FROM AN UNOPERATED MATURE FEMALE RAT. The height of the follicular epithelium and condition of the colloid are characteristic of the normal gland. ×350. Fig. 2. SECTION OF THYROID FROM A MATURE FEMALE RAT TREATED WITH THYROTROPIN. Note the increased height of the follicular epithelium, contraction and vacuolation of the colloid. ×350.

Fig. 3. *Stage 1* PSEUDO-LUTEIN BODY FROM OVARY OF AN ANIMAL TREATED WITH THYROTROPIN. ×80. Fig. 4. SECTION OUTLINED IN FIGURE 3 SHOWING APPARENT CONVERSION OF MURAL GRANULOSA INTO LUTEAL CELLS. ×350.

Fig. 5. *Stage 2* PSEUDO-LUTEIN BODY. Ovum is divided and most of mural granulosa replaced by luteal tissue. Small antrum containing follicular fluid and granulosa cells of the cumulus are still present. ×80. Fig. 6. *Stage 3* PSEUDO-LUTEIN BODY. The antrum is almost completely obliterated. Most of the granulosa, except for a small remnant of the cumulus, has apparently been transformed into luteal tissue. ×80.

Pseudo-lutein bodies, which were absent in the ovaries of the control rats, were present in those of the injected animals, but the number observed (10, 4, 4, 2, 2, and 1 in the 6 ovaries studied) was decidedly less than were formed in normal animals after similar treatment. Apparently thyroidectomy lessens the luteinizing effect of the thyrotropic preparation. The results obtained suggest that the production of pseudo-lutein bodies is due to traces of gonadotropic hormones in the thyrotropic preparation rather than to a direct or indirect effect of thyroid stimulation by the thyrotropic hormone.[5]

Normal Mature Females Injected with Thyroxin or Fed Desiccated Thyroid

In order to determine whether pseudo-lutein bodies occur in a state of hyperthyroidism, produced without possible contamination by gonadotropic hormones, the following experiments were carried out. Twelve animals were injected once daily with crystalline thyroxin[6] in the form of 1 mg. per cc. of solution in N/100 NaOH: 2 animals received 0.8 mg. (0.2 mg./4 days); 5, 1.2 mg. (0.1 mg./12 days); and 5, 1.2 mg. (0.2 mg./6 days). The estrous cycles of these animals were normal during and after the period of treatment. Histologically the thyroids showed a resting condition while the ovaries contained no pseudo-lutein bodies although many atretic follicles were observed.

Desiccated thyroid (0.25 gm.) was fed to 2, 1, and 2 rats, respectively, for periods of 12, 17 and 27 days. After 1 or 2 normal cycles all animals showed anestrus, as previously reported by Weichert and Boyd (3). The ovaries showed the same type of degeneration as was observed in the rats injected with thyroxin; histologically the thyroids were in a resting condition.

Tests for the Functional Capacity of Pseudo-lutein Bodies

It also seemed of interest to determine whether pseudo-lutein bodies possessed sufficient luteal function to: *a*) help maintain life in the adrenalectomized rat,[1] or *b*) prepare the uterus for the production of traumatic placentomata.

A. *Adrenalectomized animals; controls.* Nineteen adrenalectomized females survived an average of 11.3 (3–19) days and exhibited anestrus following 1 or 2 postoperative normal cycles. The ovaries contained old corpora lutea and many atretic follicles.

Thyrotropic injections followed by adrenalectomy. Ten animals were given 5 daily intraperitoneal injections of 0.1 cc. of thyrotropic hormone, and 7 others were given 0.2 cc., representing doses capable of producing many pseudo-lutein bodies in the normal rat (table 1). Not more than 1 or 2 pseudo-lutein bodies were observed in each of the ovaries examined. The average survival time of these animals, adrenalectomized on the day after the last injection, was 9.7 (2–15) days.

Thyrotropic injections started the day of adrenalectomy. Two groups of 6 rats each were treated as in the foregoing experiment, except that injections were begun on the day of adrenalectomy. Their average survival time was 6.3 (2–11) days, although from 10 to 16 pseudo-lutein bodies were present in the ovaries.

1% NaCl drinking solution and thyrotropic injections. Two groups of 9 rats each were adrenalectomized, given 1% NaCl drinking solution for 10 days, and during the last 5 days on NaCl therapy were injected with the same dosage as in the 2 previ-

[5] Foster and Hisaw (20) and Friedgood and Foster (21) have shown that luteinization of unruptured follicles is commonly found after treatment with follicle-stimulating and luteinizing hormones.

[6] The crystalline thyroxin was supplied by E. R. Squibb & Sons, New Brunswick, N. J.

TABLE 1. EFFECT OF DAILY INTRAPERITONEAL INJECTIONS OF THYROTROPIC HORMONE ON ESTROUS CYCLES AND OVARIES OF MATURE FEMALE RATS

Animal Number	Treatment 1/10 cc., days	Daily Vaginal Smear During Experiment	Pseudo-lutein Bodies in 1 ovary			
			Total	Stages		
				1	2	3
1	1/1/1/1/1/k	6D	10	2	2	6
2	2/2/2/2/2/k	6D	16	2	5	9
3	2/2/2/2/2/k	E/M/2D/E/M	12	3	4	5
4	2/2/2/2/2/k	6D	11	6	3	2
5	2/2/2/2/2/k	6D	13	2	2	9
6	2/2/2/2/2/-/k	7D	21	4	3	14
7[1]	2/2/2/2/2/k	P/E/4D	6	1		5
8[1]	2/2/2/2/2/-/k	P/E/M/2D/E/M	5	1	1	3
9	2/2/2/2/2/-/-/k	3D/2P/2D/P	14	4	6	4
10	1/1/1/1/1/1/1/1/1/1/k	M/8D/P/E	25	3	10	12
11	1/1/1/1/1/1/1/1/1/1/k	6D/P/3E/M	13			13
12	1/1/1/1/1/1/1/1/1/1/k	E/M/6D/P/2E	57	14	24	19
13	2/2/2/2/2/2/2/k	P/E/M/5D	18	1	4	13
14	2/2/2/2/2/2/2/k	P/E/M/5D	10	3	2	5
15	5/5/5/k	4D	14	11	2	1
16	5/5/5/k	E/M/D/P	18	3	8	7
17	2/2/2/2/2/-20 days-/k	6D, 4 cycles, D				
18	2×5 days/-24 days-/2×16 days/k	8D, 6 cycles; 8D, 2 cycles; E	37	8	15	14
19	2×5 days/-24 days-/2×17 days/k	M, 8D; 6 cycles; 15D, E	24	2	6	16

/-/=no injection; /k=killed; P, E, M, D=proestrus, estrus, metestrus, diestrus; number preceding =days in that phase of the cycle.

[1] Thyrotrophic Factor (Ayerst, McKenna and Harrison). All others Antuitrin T (Parke, Davis & Co.)

ous experiments. In this way the effect of thyrotropic hormone could be tested on animals several days after adrenalectomy. Two animals died during the first 5 days and 12 others died during the period of injections. The 18 animals survived 8.7 (3–18) days after adrenalectomy, or 5.1 (2–14) days after thyrotropic injections were started. From 1 to 7 pseudo-lutein bodies were observed in the ovaries of the injected animals.

These experiments suggest that thyrotropic hormone decreases the survival time of adrenalectomized rats despite the presence of pseudo-lutein bodies prior or subsequent to adrenalectomy. It will be noted (table 2) that the period of survival decreased in proportion to the time treatment was started before or after adrenalectomy. No difference in survival was noted, however, between the groups injected with 0.1 or 0.2 cc. daily. Since the thyroids of these animals showed histological evidence of stim-

TABLE 2. SURVIVAL TIME AFTER ADRENALECTOMY

Treatment	Number of Animals	Average and Range of Survival		Pseudo-lutein Bodies
		av.	range	
		days		
Untreated	19	11.3	3–19	
Thyrotropin before adrenalectomy	17	9.7	2–15	1–2
Thyrotropin started day of adrenalectomy	12	6.3	2–11	10–16
Adrenalectomy, NaCl and thyrotropin	18	8.7	3–18	1–7
		5.1[1]	2–14[1]	

[1] Survival from time injections were started.

Thyrotropic preparation (Antuitrin T): 0.1 or 0.2 cc. daily for 5 days.

ulation, the toxic effect of the secretion from the stimulated thyroids may have been sufficient to mask the possible beneficial effect of the pseudo-lutein bodies.[7]

B. Placentomata production after thyrotropic injections. Since the production of traumatic placentomata is an accepted criterion of luteal function, the physiological significance of the pseudo-lutein bodies was tested by the following experiment. Twelve animals, kept in anestrus for a period of 11 days by daily intraperitoneal injections of 0.2 cc. of thyrotropic hormone, had threads placed through the lumen on one uterine horn on the 5th day of treatment. No placentomata were observed in the threaded segments of the uteri, even though the ovaries contained 24 to 27 pseudo-lutein bodies on the 11th day.

This experiment indicates that the pseudo-lutein bodies induced by thyrotropic injections are not of the same functional capacity as the normal corpora lutea of pregnancy or pseudopregnancy in their ability to produce placentomata.

DISCUSSION

A direct or indirect relationship between the thyroid and female reproductive system has been suggested both clinically and experimentally. The menstrual cycles of patients with thyroid disease are very irregular and the urine of such patients may contain gonadotropic principles (23) or give a positive Friedman pregnancy test (24). Experimentally the hyperthyroid condition may interrupt the normal reproductive functions of the nonpregnant or pregnant rat (1, 2, 3). It has not been established whether the changes in the female reproductive system in hyperthyroidism are due to abnormal functioning of the pituitary caused directly or indirectly by the increased metabolism.

The increased metabolic utilization of the estrus-producing hormones has been reported to be the probable cause of retention of the luteal phase of the cycle. If this is so, then the corpora lutea, retained after feeding desiccated thyroid in amounts sufficient to produce pseudopregnancy, are apparently of the same functional capacity as normal corpora lutea in their ability to prepare the uterus for the production of traumatic placentomata (3). The pseudo-lutein bodies, however, appear to be artificially induced corpora lutea resulting from the presence of gonadotropic substances in the thyrotropic preparations. Artificially induced corpora lutea have been found not to produce progestin by the placentomata reaction (25).

The formation of pseudo-lutein bodies apparently was not due to a pure hyperthyroid condition since they were formed in the ovaries of thyroidectomized rats treated with thyrotropic preparations. The functional significance of the pseudo-lutein bodies has not been determined. They will neither help maintain life in the adrenalectomized rat nor prepare the uterus for the production of traumatic placentomata.

SUMMARY

Injections of thyrotropic preparations into normal immature and mature rats or thyroidectomized mature rats produced luteinization in unruptured follicles. The lutein tissue was apparently derived from the mural granulosa. Since this phenomenon did not occur after injections of thyroxin or oral administration of desiccated thyroid, it is concluded that the formation of luteinized follicles (pseudo-lutein bodies) was due to gonadotropic substances in the thyrotropic preparations and not to a hyperthyroid condition.

Pseudo-lutein bodies were not of the same functional capacity as normal corpora

[7] Kendall (22) and others have found thyroxin to be toxic to adrenalectomized rats.

lutea since they did not prepare the uterus for the production of traumatic placentomata or help maintain life in the adrenalectomized rat. The secretion from the thyrotropic-stimulated thyroids apparently was toxic to the adrenalectomized rats.

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THE EFFECT OF THYROIDECTOMY ON LACTATION IN THE ALBINO RAT

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PRESENT UNCERTAINTY concerning the relationship of the thyroid to lactation is indicated by the discrepancies in reported experimental results. In rabbits (1) and in a woman (2) an increase in lactation was said to occur during hypothyroidism. On the other hand, others report that thyroidectomy diminishes and alters lactation in the goat (3–7), the cow (8), the rat (9–12) and man (13, 14, 15). Still other investigators could find no change in lactation after thyroidectomy in the rat (16, 17) and in the mouse (18).

Hyperthyroid and thyroidectomized cows given thyroid hormone show an increased lactation (19, 20, 21). Reports that thyroid feeding will decrease lactation are based on some questionable clinical data (22, 23, 24). Siegert (25) states that thyroid feeding inhibits lactation in guinea pigs, but histologically, his photomicrographs do not seem to bear this out.

The concensus of the above reports is that *a*), the thyroid is not essential for lactation, but that its absence causes a decreased and altered secretion of milk; and *b*), that hyperthyroidism, induced by optimal doses of thyroid causes an increase in lactation, but that an excess of thyroid reduces it. Nelson and Tobin (16) and Nelson (17) declared that thyroidectomy had no effect upon lactation in the rat; Preheim (12) said that it caused a "slight," and Folley (11) that it caused a "moderate" decrease in lactation in this animal.

METHODS

In view of these conflicting statements, virgin albino rats, weighing 200 gm. each and about 140 days old, were used in the following experiments. Each rat was kept in a separate cage. Thyroidectomy was performed under ether anesthesia *a*), before conception, *b*), during gestation and *c*), in most cases immediately after delivery. All litters were reduced to 5 within a day of birth, but frequently, in the experimental group, death of some of the young further reduced this number. The young were weighed daily and the weights used as an indication of milk production.

RESULTS

Two groups of animals were used in these experiments. *Group I* was fed on a diet of cracked-corn, 'scratch,' dog-biscuit, lettuce and butter. Of this group, 10 were thyroidectomized immediately after delivery and 9 were sham-operated controls. The 10 thyroidectomized mothers (fig. 1, *C*) lost weight following the operation, but the control mothers (fig. 1, *A*) did not. As seen in figure 1*B*, the young of the thyroidectomized mothers gained but slightly in weight and many of them died within the first 16 days, while the young of the control mothers gained weight steadily. This suggested that there was a marked decrease, and in some cases probably a complete

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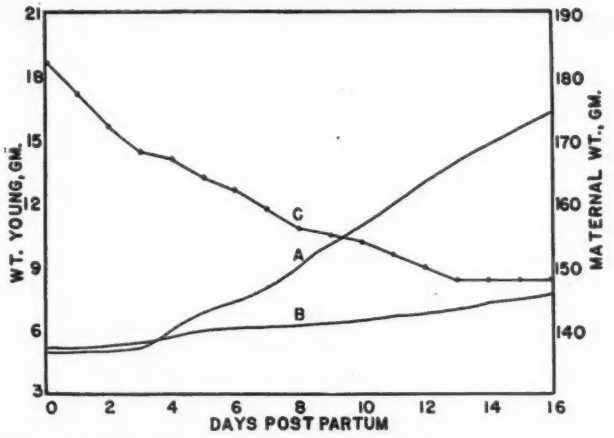

Fig. 1. Graph of data obtained from *Group I*. Weights of the young are plotted as average weight of each young per litter and the weights of mothers are plotted as average weight per mother. *A*. Weights of control young (9 litters); *B*. Weights of young of thyroidectomized mothers (10 litters); C. Weights of ten mothers thyroidectomized immediately postpartum.

cessation of lactation following thyroidectomy, but the loss of maternal weight of those which were thyroidectomized seemed to vitiate the results, and it was necessary to repeat the experiment.

The experiments with *Group II* were made a year later. These rats were fed on a balanced diet of corn meal, 62.5 per cent; casein, 10 per cent; linseed-oil cake meal, 10 per cent; sardine oil, 3.5 per cent; alfalfa, 2 per cent; bone-ash, 0.5 per cent; and salt, 0.5 per cent. The mothers in this group did not lose weight following thyroidectomy, their weights closely paralleling those of the controls. The mothers thyroidectomized at various times before delivery maintained their body weight during lactation (fig. 2, *D*, *E* and *F*).

Any effect upon the weight of the young in this group can hence be assumed to be due to thyroidectomy of the mothers. Nine mothers were thyroidectomized immediately after delivery, and 5 were sham-operated controls. Although some of the young of these thyroidectomized mothers died the rest gained weight (fig. 2, *B*), though not as much as the controls (fig. 2, *A*). However, the young of both the control and thyroidectomized animals gained more than the young of comparable animals in *Group I*.

Fourteen thyroidectomized females, including the ones used in the above group, were mated, 8 becoming pregnant. Six females, thyroidectomized on the 14th day of gestation, were added to these. Eight delivered and nursed their young but lost no weight. These 8 included 5 which had been thyroidectomized before pregnancy, and 3 which had been operated on at the 14th day of gestation (fig. 2, *F*). The young gained weight, the increase closely paralleling that of the young whose mothers were thyroidectomized immediately post partum, although this gain was less than in the controls (fig. 2, C). A reduction of lactation hence was indicated in both of these groups of thyroidectomized rats.

DISCUSSION

The reason for the variation in the degree of inhibition of lactation after thyroidectomy in *Groups I* and *II* seems to lie in the differences in the nutritional state

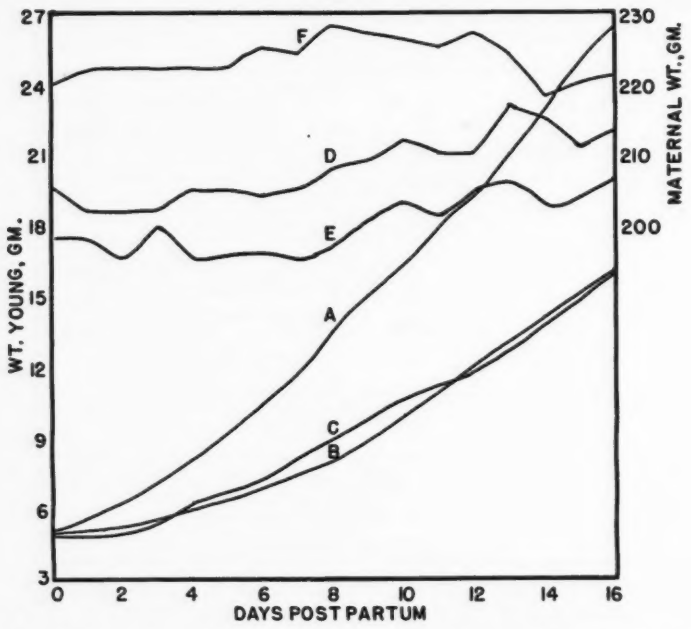

Fig. 2. Graph of data on *Group II*. Weights of the young are plotted as average weight of each young per litter and that of the mothers is plotted as average weight per mother. *A*. Weight of control young (5 litters); *B*. Weight of young of mothers thyroidectomized postpartum (9 litters); *C*. Weight of young of mothers thyroidectomized at various times before delivery (8 litters); *D*. Postpartum weight of five control mothers; *E*. Postpartum weight of five mothers thyroidectomized after delivery; *F*. Postpartum weight of five mothers thyroidectomized at various times before delivery.

of the mothers. It is to be expected that mothers that eat little and lose weight during lactation will give less milk than those that eat more and suffer no loss of weight. The reason for one group losing weight after thyroidectomy when the other one did not is not clear.

Differences in diet would appear to be the most obvious factor. However, during a previous experiment upon 102 thyroidectomized male rats weighing 150 to 250 gm. (26) which had been kept on the same diet and records made of the duration of the operation, the degree of trauma, the amount of bleeding and the rate of recovery from the anesthesia, no correlation was established between the severity of the operative procedure (in the absence of complications) and the postoperative loss in weight (fig. 3). There were marked differences in the losses in weight between the groups although the diet and operative procedure were identical. Other investigators have commented upon the variation in loss of weight (27, 28, 29). The importance of recording the nutritional state of experimental animals after thyroidectomy, as well as after any experimental procedure, needs further emphasis.

In order to check the completeness of the thyroidectomy, 10 of the mothers in *Group II* were killed at the end of the experiment, the tracheae removed, sectioned serially, stained, and examined carefully. In 2, no thyroid tissue was found. In 8 in which thyroid remnants were present, the tissues were reconstructed graphically and measured. The estimated volume of these fragments varied from 0.011 cmm. to 2.592 cmm. with an average of 0.797 cmm. but all of these animals had shown evidence of

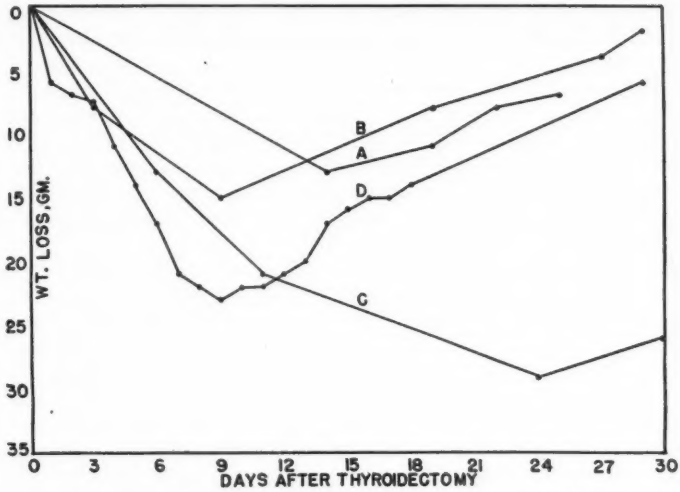

Fig. 3. AVERAGE LOSS OF WEIGHT IN GRAMS of 102 male rats following thyroidectomy. *A. Group I*, 30 rats operated in Jan., 1937; *B. Group II*, 26 rats operated in March, 1937; *C. Group III*, 23 rats operated in July, 1937; *D. Group IV*, 23 rats operated in Oct., 1937.

hypothyroidism; they appeared lethargic and the hair was rough and slightly pigmented. No correlation was noted between the volume of the thyroid remnants and the degree of suggested inhibition of lactation.

Complete thyroidectomy is extremely difficult in the rat, for even in those operated upon most carefully, there may be a few isolated follicles in the ventral portion of the peritracheal fascia or immediately surrounding the recurrent laryngeal nerves, at the superior pole of the thyroid. However, it seems that the presence of these aberrant follicles did not appreciably affect the results of our experiments.

Parathyroid tissue was noticed in the sections of only 2 tracheae, but this does not preclude its presence elsewhere in the neck. However, the possibility that hypoparathyroidism affected the results must be considered. Although the parathyroid glands are usually removed incidentally in thyroidectomy in rats, the animals ordinarily show no evidence of hypoparathyroidism, and the results observed under these circumstances have been assumed to be due solely to the removal of the thyroids. It is usually agreed (27, 30) that the removal of the parathyroids during thyroidectomy is not of great importance except in pregnancy and lactation, during which the increased calcium and phosphorus metabolism frequently produces tetany in thyroparathyroidectomized rats. Chandler (30) found that parathyroidectomized rats often are lethargic and tetanic during the last 5 days of pregnancy, that delivery may be prolonged and difficult, and that these signs increase during lactation. When more than 5 young were nursed they usually were smaller than average. Bodansky and Cooke (31) found not only that thyro-parathyroidectomized rats were irritable at term but also that some went into tetany and died during delivery. Some of our observations confirm this. Three of the mothers of *Group I* and one of *Group II*, thyroidectomized after delivery, showed signs of tetany during lactation, lost weight, and did not nurse their young. Six of the thyroidectomized mothers which had been operated on prior to delivery showed signs of lethargy and depression 2 days before term. Two of these died in dystocia, one apparently chewing her own tail to shreds

just before her death. Since one delivered a dead litter and 3 delivered living young which died during the next 2 days, these animals were not included in our lactation series.

It would appear then that in these experiments hypoparathyroid rats were recognized and excluded, thus making it unlikely that parathyroidectomy significantly affected our results. Moreover, Folley (11) found that the administration of parathormone did not increase the milk output of thyroidectomized rats. Nevertheless, studies of calcium and phosphorus metabolism would have been of value in determining the presence and the degree of subclinical states of parathyroid deficiency in these animals.

Restoration of normal lactation in thyroidectomized rats was attempted by giving 5 rats, which had been thyroidectomized immediately post partum, 0.120 gm. of desiccated thyroid daily. Three of the 5 litters of the young began to gain more rapidly than those of untreated, thyroidectomized animals, but on the fifth day the treated mothers became extremely hypertonic and refused to nurse their young; the latter lost weight rapidly and died within two days. The experimentally produced hyperthyroid condition of the mothers apparently kept them from nursing.

Folley was unsuccessful in increasing lactation in thyroidectomized rats by giving 0.1 mg. of sodium-thyroxine daily to one group and 0.25 mg. of sodium-thyroxine together with 1 U of parathormone every fourth day to another. The gain in weight of the young observed by us may have been due to other factors, but since 3 of the animals lactated until symptoms of hyperthyroidism kept them from nursing their young, the possibility of an overdose of thyroid must be considered; it has been shown (32) that thyroidectomized rats are more sensitive to thyroxine than normal rats. Replacement therapy, apparently, must be attempted with doses of thyroxine lower than the above, to obtain an optimal effect on lactation.

As do those of most observers, our findings show that thyroidectomy diminishes lactation in the rat, our data corresponding most closely with those of Folley. The moderate decrease in lactation found by Preheim (12) and the failure reported by Nelson and Tobin (16) and Nelson (17) to effect any alteration in lactation following thyroidectomy is difficult to understand.

We (33) noted that histologically the state of the mammary glands of the thyroidectomized rats was similar to that of the controls, at the time of parturition, and that they developed normally during pregnancy. During lactation, however, considerable stress is placed upon the general body metabolism, and thyroidectomy then causes a lowering of this metabolic activity. Furthermore, the marked changes noted in the pituitary gland after thyroidectomy prompt one to postulate, as others have done, a limitation of the output of prolactin; the restoration of lactation to normal in thyroidectomized rats, through administration of it, would be a confirmation of this.

SUMMARY AND CONCLUSIONS

Although the thyroid gland does not seem to be essential for lactation in the rat, the lesser increase in the weight of the young suggests that thyroidectomy immediately after delivery, during gestation, or before conception causes a diminution in lactation.

Thyroidectomy before conception or during gestation does not inhibit the evolution of the mammary glands to a condition which appears histologically comparable to that of the normal, active, lactating state. This work lends support to the suggestion that dimunition of lactation after thyroidectomy probably is due to effects upon the pituitary gland affecting the formation of prolactin.

Thyroid replacement therapy in thyroidectomized rats suggests that lactation was increased thereby.

The variations in the loss of weight of mature male and female rats following thyroidectomy could not be explained.

I wish to express my sincere appreciation to Dr. A. W. Meyer for his valuable aid and considered criticism during the course of this investigation.

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PHYSIOLOGICAL REACTIONS OF THE THYROID-STIMULATING HORMONE OF THE PITUITARY

I. ITS INACTIVATION BY EXPOSURE TO THYROID TISSUE IN VITRO[1]

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MANY ATTEMPTS have been made to demonstrate the thyroid-stimulating hormone in the urine of patients with thyroid disease. More recent studies include those of Hertz and Oastler (1), Cope (2), Rawson and Starr (3), Starr et al. (4) and Jones (5). Hertz and Oastler found a thyroid-stimulating substance in the urine of 9 myxedematous patients but were unable to demonstrate any hormonal effect in the urine of normal or thyrotoxic individuals. Both Cope and Jones reported that they were not able to demonstrate the hormone in the urine of myxedematous patients. Rawson and Starr found demonstrable amounts of a thyroid-stimulating substance in the urine of 21 normal medical students. They found a strongly positive thyroid-stimulating effect in the urine of three men who had had total thyroidectomies for heart disease and in the urine of a majority of nonmyxedematous patients whose basal metabolic rates varied between -34 and -17. No evidence of thyroid-stimulating hormone was found in the urine of 12 of 14 thyrotoxic patients.

While considering such observations, it is interesting to refer to the hypotheses advanced by Marine (6) and by Means (7). Marine suggested that "the thyroid and anterior pituitary glands are delicately balanced" and that "any deficiency in thyroid secretion quickly stimulates the pituitary to produce more thyrotropic hormone; and conversely, supplying the thyroid secretion reduces anterior pituitary activity." More recently Means has suggested that there might prove to be a functional equilibrium between the thyroid gland and the anterior pituitary gland each acting on the other through the concentration of its hormone in the blood. If these theories should prove correct, one might explain the presence of the thyroid-stimulating hormone in the urine of hypothyroid individuals as being due to a compensatory action of the pituitary. The absence of any demonstrable hormone in the urine of thyrotoxic patients might be due to a suppression of the thyroid-stimulating factor of the pituitary because of an increased amount of some thyroid hormone in the circulating blood. However, another point of view is possible, namely, that the absence of any thyroid-stimulating hormone in the urine of hyperthyroid patients might be due either to the altering or retention of the hormone by the hyperplastic thyroid gland. The purpose, therefore, of our studies has been to investigate in vitro the possibility of the thyroid gland having a preferential altering effect on the activity of its stimulator. Two recent investigations lend support to this theory. In 1934 Loesser (8) suggested that the thy-

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roid-stimulating hormone might be removed from the circulating blood by the thyroid gland. He injected 3000 units of TSH intravenously into intact rabbits and recovered the hormone from the circulating blood for only one hour. In the blood of thyroidectomized animals which had been treated in a similar manner he was able to demonstrate a thyroid-stimulating effect for 7 hours. In the midst of our present studies, Seidlin (9) reported that he had demonstrated the hormone in the urine of thyroidectomized guinea pigs after the injection of large doses of similar hormone. He was not able to recover any thyroid-stimulating substance from the urine of intact guinea pigs treated in a similar manner. He also observed that the thyroid-stimulating hormone was partly removed from solution when it was brought into contact with a saline suspension of thyroid tissue.

METHODS

Tissue culture techniques were resorted to for this study. Special roller bottles devised in this laboratory (10), were coated with chicken plasma. The bathing fluid consisted of 15 cc. of Tyrode's solution (11) which contained the thyroid-stimulating hormone[2] in varying concentrations (¼, ½ and 1 Junkmann Schoeller (12) guinea pig unit per cc.). The thyroid of a normal adult rabbit (60–100 mg.) was sliced and explanted in such a bottle; and rotated 15 revolutions per hour in an incubator at 38° C. At the end of a three-day period the medium was withdrawn and assayed for thyroid-stimulating effect on two guinea pigs.

As a control, media containing the hormone in a concentration equivalent to ¼ of a unit per cc. were incubated in plasma coated bottles containing no tissue. Other control studies included three-day incubations of other tissue explants with media containing the hormone in a concentration of ¼ unit per cc. The control tissues included rabbit adrenals, kidneys, livers, ovaries, pancreas, stomach mucosa, testes and thymus; rat thymus and lymph nodes.

Female guinea pigs weighing between 180 and 210 gm. were used for assaying the thyroid-stimulating hormone. The animals were injected with one cc. of the test medium daily for 3 days and were killed on the fourth day by ether inhalation. The thyroids were removed immediately and fixed in 10 per cent neutral formalin for 24 hours. After dehydrating in the various concentrations of alcohol and in chloroform, the tissues were embedded in paraffin (melting point between 56 and 58° C.). Sections were cut at 6 micra and stained with hematoxylin and eosin. The microhistometric method described by Starr and Rawson (13) was used in evaluating the hormonal effect. The sections were examined under the oil immersion lens. Each examined section was covered systematically from end to end in parallel pathways across the center of the gland until 200 acini had been studied. Each acinus in the path of crossing having a lumen large enough to present a definite wall and cut cross-sectionally was included in the study. From each acinus one representative cell was chosen and its height determined by means of an ocular micrometer. Frequency curves were plotted from the observed measurements and the mean acinar cell height of each examined thyroid was derived. To determine the normal responses of guinea pig thyroids to the pituitary extracts used in this investigation, 4 groups of animals were given 3 daily injections of the hormone in dilutions equivalent to ⅛, ¼, ½ and 1 Junkmann Schoeller units. The thyroids of these animals were fixed and sectioned. The mean acinar cell heights of their thyroids were determined and compared with those of 22 untreated guinea pigs. The average mean cell height of each group of animals is recorded

[2] The hormonal preparations were generously supplied by Dr. E. A. Sharpe of Parke, Davis and Co., Detroit, Michigan and by Dr. Frederic Fenger of the Armour Laboratories, Chicago, Illinois. The TSH which we used is, of course, not pure but we could not find evidence of adrenotropic hormone though it probably contained some gonadotropic hormone.

TABLE 1. RESPONSE OF MEAN ACINAR CELL HEIGHTS OF GUINEA PIG THYROIDS TO INCREASING CONCENTRATIONS OF THYROID STIMULATING HORMONE

Concentration of TSH (in Junkmann Schoeller units per cc.)	No. of guinea pig assays	Thyroid mean acinar cell heights (in micra)	
		Range	Average
None	22	2.4–2.6	2.5
1/8	4	3.0–3.4	3.3
1/4	4	4.1–4.3	4.2
1/2	4	4.7–4.9	4.8
1	4	5.3–5.6	5.5

in table 1, which demonstrates a consistent response to each concentration of the hormone and presents a graphic increase in the mean acinar cell height of the thyroid in response to increasing doses of the extracts. It also provides a standard with which the responses of experimental animals can be compared.

RESULTS

Inactivation of the thyroid-stimulating hormone (TSH) by thyroid tissue. It was consistently observed that the bathing media, originally containing the thyroid-stimulating hormone in concentrations equivalent to ¼ and of ½ units per cc., had lost completely their thyroid-stimulating effects following incubation with thyroid tissue. Likewise, media which originally contained TSH in a concentration equivalent to 1 unit per cc. were found to have lost about ⅞ of their activity. The mean acinar cell heights of animals which had been treated with the hormone-containing media, after they had been exposed to in vitro thyroid tissue, are recorded in table 2.

Because of the prolonged exposure in these experiments, another observation was made with shorter contact periods between the TSH and thyroid tissue. Three normal rabbit thyroids averaging 80 mg. in weight, were sliced and explanted into three separate roller bottles. Each explanted gland was bathed in 15 cc. of Tyrode's solution which contained ½ unit of TSH per cc. An aliquot of the medium was removed from each bottle and pooled at 2, 6, 12, 24, 48, and 72-hour intervals after the explanting. Each pooled specimen was assayed on 2 guinea pigs. The pooled media removed at the end of 2 hours contained enough active hormone to produce mean acinar cell heights of 2.9 and 3.1 micra, contrasted with an average control MACH of 2.5 micra. None of the pooled media specimens removed after 6 hours contained any demonstrable hormonal effect. The guinea pig thyroid mean acinar cell heights obtained from assays of these media averaged 2.4 micra.

Effect of control tissues on the activity of the thyroid-stimulating hormone. Media containing the hormone in a concentration equivalent to ¼ unit per cc. did not lose their thyroid-stimulating effect when incubated without tissue in plasma-coated

TABLE 2. INACTIVATION OF THE THYROID-STIMULATING HORMONE BY THYROID, THYMIC, AND LYMPH NODE TISSUES

Concentration of TSH (units per cc.)	Tissue to which the hormone was exposed	No. of subsequent guinea pig assays	Thyroid mean acinar cell heights[1] (in micra)	
			Range	Average
1/4	Thyroid	32	2.3–3.0	2.5
1/2	Thyroid	18	2.3–2.6	2.4
1	Thyroid	8	3.0–3.1	3.0
1/4	Thymus	10	2.4–2.6	2.5
1/2	Thymus	10	2.9–3.2	3.0
1/4	Lymph node	10	2.3–2.6	2.5
1/2	Lymph node	10	3.0–3.3	3.1

[1] The thyroid mean acinar cell heights of guinea pigs injected with media which originally contained TSH in the stated concentrations and which were first incubated with the indicated tissues.

bottles. Of greater significance were the observations that the hormone when incubated with control-tissues which included explants of adrenals, kidneys, livers, ovaries, pancreas, parathyroids, spleens, stomach mucosae and testes did not lose any activity. The mean cell heights of assays of these control media are listed in table 3.

There were however, two control tissues which reduced the thyroid-stimulating effect of the hormone in their bathing media. They were thymus and lymph nodes, explants of which weighed 100 to 150 mg. Media containing the equivalent of ¼ of a unit of TSH per cc. were found to contain no thyroid-stimulating effect following a 3-day incubation with thymic tissue or explants of lymph nodes. The same tissues were found to have removed about ¾ of the activity of a solution which contained ½ unit of the hormone per cc. The results of these assays are recorded in table 2.

TABLE 3. EFFECT OF CONTROL TISSUES AND OF INCUBATION WITHOUT ANY TISSUE ON THE ACTIVITY OF THE THYROID-STIMULATING HORMONE

Concentration of TSH (unit per cc.)	Tissue to which the hormone was exposed	No. of subsequent guinea pig assays	Thyroid mean acinar cell heights[1] (in micra) Range	Average
1/4	No tissue	8	4.1–4.3	4.2
1/4	Adrenal	8	4.0–4.4	4.1
1/4	Kidney	8	4.0–4.3	4.1
1/4	Liver	8	3.9–4.1	4.0
1/4	Ovary	8	3.9–4.1	4.1
1/4	Pancreas	8	3.9–4.2	4.1
1/4	Parathyroid	6	4.1–4.3	4.2
1/4	Spleen	6	4.1–4.2	4.2
1/4	Stomach mucosa	4	4.1–4.2	4.1
1/4	Testis	4	4.0–4.2	4.1

[1] The recorded mean acinar cell heights are of animals treated with media containing TSH which had been incubated with the indicated control tissues or had been subjected to incubation without tissue.

The thyroid explants when exposed to the stimulating hormone did not show any evidence of growth. This might have been due to the fact that the tissues were adult and to the fact that the bathing medium was not one likely to promote growth. However, sections made of the thyroid explants after their exposure to the pituitary hormone did show cellular hypertrophy. In an attempt to determine the effect of tissue viability on the effects described, we destroyed the viability of otherwise normal thyroids by temperature changes before explanting. Three rabbit thyroids were frozen in CO_2 ice for one hour and three other thyroids were boiled in water for 30 minutes before they were explanted. All of these tissues were explanted separately and were bathed in media containing the equivalent of ¼ unit of TSH per cc. The frozen thyroids retained their ability to inactivate the hormone. Six guinea pigs treated with the media in which the frozen thyroids had been bathed had thyroid mean acinar cell heights which averaged 2.5 micra. However, boiled thyroids were unable to remove the thyroid-stimulating effect from their bathing media. The thyroid mean acinar cell heights of 6 guinea pigs treated with media in which the boiled thyroids had been incubated averaged 4.1 micra.

Effect of thyroxin on the activity of TSH. Since there is considerable evidence that the thyroid hormone exercises a depressing effect on the action of the pituitary's thyrotropic hormone (14, 15, 16), we did the following experiment to determine whether the described changes in the action of the pituitary hormone might be due to thyroxin, which might have been washed out of the explants. Tyrode's solutions containing TSH in concentrations of ¼ or of ½ units per cc. were incubated for 3 days with a .04 mg. per cc. concentration of thyroxin and assayed for thyroid-stimulating hormone. Such assays demonstrated a partial loss of thyroid-stimulating

effect from the solution containing thyroxin. Thyroxin solutions containing the pituitary hormone in a concentration of ¼ unit per cc. produced mean cell heights which averaged 3.1 micra. Similar solutions containing ½ unit of TSH per cc. produced mean cell heights which averaged 4.1 micra. However, one cannot be too enthusiastic about this observation, as several possible errors are inherent in the technic. The addition of the thyroxin solution to the media with a drop in pH to 7.6, probably produced in undertermined reduction in the amount in solution. The observation ought and will be repeated using thyroglobulin and diiodotyrosine.

DISCUSSION

These observations indicate that thyroid tissue, when incubated in vitro with pituitary-extracted thyroid-stimulating hormone, inactivates its stimulator. These findings when considered with those which were reported by Loesser (8) and by Seidlin (9) suggest that the reports of demonstrable amounts of thyroid-stimulating substances in the urine of athyreotic and hypothyroid individuals might be explained as the failure of a nonfunctioning thyroid to bind the pituitary hormone. They might also be used to explain the reports of the absence of any thyroid-stimulating substances in the urine of classically thyrotoxic patients as being due to the complete inactivation of pituitary secreted thyrotropic substances by an overactive thyroid.

Since the hormone in concentrations of ¼ and of ½ units per cc. was completely inactivated by normal rabbit thyroid tissue, and only about 7/8 of the thyroid-stimulating effect was removed from a medium containing the hormone in a concentration of 1 unit per cc., it appears that there is a limited amount of neutralizing power for this substance in normal thyroid cells.

In the light of present chemical knowledge it is difficult to explain the mechanism of inactivation of the thyroid stimulator by its exposure to thyroid or thymic tissue or to lymph nodes. However the data suggest several possible mechanisms. One suggestion is that though no prosthetic group has been demonstrated in the proteins of any anterior pituitary hormone, these observations might be explained on the basis of the presence of such a group. Such a prosthetic group might be removed from the pituitary hormone by an enzyme peculiar to the thyroid, thymus and lymph nodes, or it might be bound through union of the TSH with a substance which is liberated by these organs.

In these observations, the inactivating reaction takes place in the bathing fluid and of course they do not prove that such a reaction takes place in the intact gland. It must be remembered however, that using tissue slices exposes the media to liberated intracellular substances. In order to explain the local growth responses of the thyroid to the pituitary hormone, it would appear likely that the reaction in life occurs in the gland itself.

Significantly, none of the control tissues excepting the thymus and lymph nodes was able to alter the activity of the thyroid-stimulating hormone. Although these two tissues have a qualitative effect on the TSH similar to that of the thyroid, their quantitative effects are less. Various workers notably Marine and co-workers (17), have called attention to the hypertrophy of the thymicolymphatic tissues in thyrotoxicosis. It is interesting that the very tissues which hypertrophy in hyperthyroidism react similarly in these experiments. This suggests that these observations have fundamental biological importance and bear a relationship to conditions found in thyroid diseases.

SUMMARY

A method has been described which uses in vitro technic for determining the reaction of the thyroid-stimulating hormone with body cells. This method might also

be adapted to the study of other hormones of the anterior pituitary gland.

It has been observed that the thyroid-stimulating effect of a pituitary extract is removed following its exposure to normal thyroid tissue.

Similarly, lymph nodes and thymic tissue when bathed in solutions of thyroid-stimulating hormone also decrease the hormone's activity, though to a lesser degree.

Other control tissues which include adrenals, kidneys, ovaries, pancreas, parathyroids, spleens, stomach mucosae, and testes do not remove the activity of the hormone. The reactions of the thyroid-stimulating hormone are limited, therefore, to relatively few cell types.

Theoretical implications of these observations have been discussed.

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BODY WEIGHTS AND WORK PERFORMANCE OF ADRENALECTOMIZED RATS TREATED WITH PROGESTERONE, Δ^4-17 A-METHYL-D-HOMO-ANDROSTENE-17 A-OL,-3, 17-DIONE, AND 6-HYDROXY-11-DESOXYCORTICOSTERONE DIACETATE

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PROGESTERONE IS CAPABLE of maintaining the life of adrenalectomized rats (1) and other animals. Ruzicka and Meldahl (2) reported the synthesis of a compound which was first described as 17-hydroxy-progesterone and it was stated (3) that Professor E. Laqueur had found the substance to be approximately as effective as corticosterone in maintaining the life of adrenalectomized dogs. Subsequently, Ruzicka and Meldahl (4) found that rearrangement had occurred during synthesis of the compound and that its structure is that of Δ^4-17a-methyl-D-homo-androstene-17a-ol-3, 17-dione. It is, therefore, not a member of the progesterone or corticosterone series. Ehrenstein (5) prepared 6-hydroxy-11-desoxycorticosterone diacetate by partial synthesis. This substance is a position isomer of corticosterone.

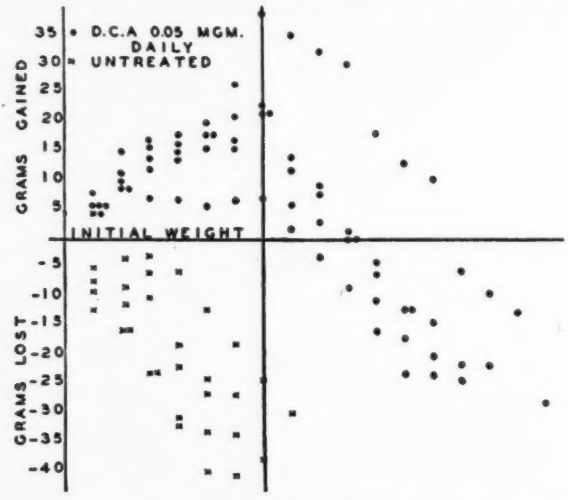

Fig. 1. BODY WEIGHTS OF ADRENALECTOMIZED RATS. Initial weight 180 ± 2 gm. Treated with 11-desoxycorticosterone acetate (D.C.A.).

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These three compounds have been examined for their effect on the body weight and the work performance of adrenalectomized rats.

In addition, Pfiffner and North (6) isolated 17 (β)-hydroxy-progesterone from adrenal glands and Ehrenstein and Stevens (7) prepared the acetate of 6-hydroxy-progesterone by partial synthesis. These two compounds are position isomers of 11-desoxycorticosterone. Small amounts of these compounds were examined for effect on the work performance of adrenalectomized rats. As it is uncertain whether additional amounts of these two compounds will be prepared the incomplete results are mentioned.

TABLE 1. WORK PERFORMANCE OF RATS IMMEDIATELY FOLLOWING ADRENALECTOMY

Dose (twice daily)	Substances		
	Progesterone	Ruzicka compound[1]	6-hydroxy-11-desoxy-corticosterone diacetate
mg.	*Rec. Rev.*[2]	*Rec Rev.*[2]	*Rec. Rev.*[2]
0.25			4175
			4781
0.50			5825
			5242
1.00	3719	3499	4053
	5495	3003	2463
2.00	5344	2904	3363
	3661	3172	
5.00	2645	2671	
	5337	3895	
10.00	4273	1208	
	6541	4081	
20.00	6481		
	2941		
45.00	3524		
	4038		

[1] Δ^4-17a-methyl-D-homo-androstene-17a-ol-3,17-dione.

[2] Work is expressed in terms of recorder revolutions (Rec. Rev.). Each recorder revolution is equivalent to approximately 400 gram-centimeters of work.

The range in performance of 10 untreated animals was 2452 to 6995 recorder revolutions.

The range in performance of 10 sham-operated animals was 15,485 to 24,742 recorder revolutions for a 24-hour period of work.

Doses of 0.25 mg. or more of 17-hydroxy-11-dehydrocorticosterone consistently increase the amount of work above the range of untreated adrenalectomized rats.

Male rats of the Sprague Dawley strain which weighed approximately 180 gm. were used in these experiments. The diet was Purina dog chow. Bilateral adrenalectomies were performed in one stage under ether anesthesia. The test substances were dissolved in sesame oil and administered by subcutaneous injection twice daily. The rats were *a*), subjected to the work test immediately following operation (table 1), *b*), maintained for 7 days and then subjected to the work test (table 2) or *c*), they were treated for 7 days and then maintained untreated, without testing the ability to work, to determine the weight curves (fig. 1, 2 and 3).

For the work test each animal was anesthetized with phenobarbital sodium. The left gastrocnemius muscle was weighted with 100 grams and stimulated to contract 3 times per second. Each animal received 5 cc. of water by subcutaneous injection

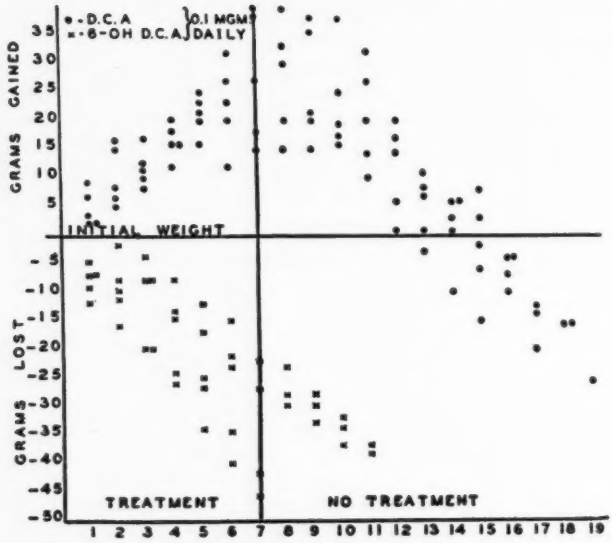

Fig. 2. Body weights of adrenalectomized rats. Initial weight 180±2 gm. Treated with 11-desoxy-corticosterone acetate (D.C.A.) and 11-desoxy-6-hydroxy-corticosterone diacetate (6-OH-D.C.A.).

twice daily for as long as the animal continued to work. In those animals which were subjected to the work test immediately following operation stimulation was continued until the muscle ceased to respond or for a maximum period of 24 hours.

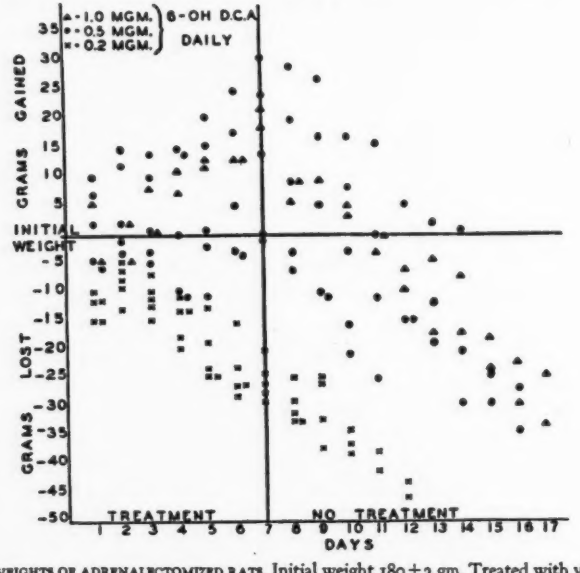

Fig. 3. Body weights of adrenalectomized rats. Initial weight 180±2 gm. Treated with various dosage levels of 11-desoxy-6-hydroxy-corticosterone diacetate (6-OH-D.C.A.).

TABLE 2. BODY WEIGHT AND WORK RECORDS OF ADRENALECTOMIZED RATS AFTER 7 DAYS OF TREATMENT (initial weight 180±2 gm.)

Daily Dose	Progesterone		Δ^4-17a-methyl-D-homo-androstene-17a-ol-3, 17-dione	
	Weight	Work	Weight	Work
mg.	gm.	Rec. Rev.*	gm.	Rec. Rev.*
0.125			174	1515
			152	1216
			150	1658
			162	1745
			134	60
0.25			173	2254
			dead	
			140	846
			160	1011
			158	1329
0.50	156	871	163	1543
	175	2312	175	1498
	177	1048	177	1195
	148	1023	160	750
	139	352	156	783
1.00	170	894	177	1656
	181	2108	141	120
	140	136	159	660
	171	758	152	512
	134	18	172	2513
2.00	160	1605	162	819
	150	1423	150	883
	158	968	142	799
	170	852	152	118
	186	1365		
5.00	173	2324		
	185	3970		
	174	1290		
	180	5311		
	182	2160		
10.00	198	7660		
	200	4761		
	196	3852		
	204	5415		
	195	5361		

* Work is expressed in terms of recorder revolutions (Rec. Rev.) Each recorder revolution is equivalent to approximately 400 gram-centimeters of work.

Ten untreated rats under similar conditions showed a range in body-weight of 140 to 177 grams with an average of 153 grams; the amounts of work averaged 1106 recorder revolutions with a range of 123 to 2565.

Six sham-operated rats under similar conditions showed a range in body-weight of 191 to 226 gm. with an average of 212 gm.; the amounts of work averaging 112,181 recorder revolutions with a range of 101,792 to 121,200.

Those animals which were treated for 7 days prior to the beginning of the work test were made to work until the muscle ceased to respond. The details of the methods have been described (8, 9).

When progesterone was administered in large doses to adrenalectomized rats (table 2) they gained weight. Those animals which showed a positive weight response performed more work than untreated animals but their ability to work was very

poor compared to that of rats treated with corticosterone and similar compounds (10) and to that of sham-operated animals. The administration of progesterone to those rats which were made to work immediately following adrenalectomy failed to enhance the work performance even when massive doses were administered. The limited amount of Δ^4-17a-methyl-D-homo-androstene-17a-ol-3, 17-dione available did not permit testing amounts larger than 2 mg. daily for effect on weight and work (table 2) and 10 mg. per dose in the acute test (table 1). By both criteria the doses tested were ineffective.

In the acute work test (table 1) the administration of 6-hydroxy-11-desoxycorticosterone diacetate up to 2 mg. per dose was ineffective. Because there is a relation between the work test and the response of diabetic animals, one partially depancreatized rat was treated with 2 mg. of this substance daily for 5 days without inducing a glycosuria. This animal developed a severe glycosuria when 1 mg. per day of 17-hydroxy-11-dehydrocorticosterone was administered for a similar period. However, the compound, 6-hydroxy-11-desoxycorticosterone diacetate had a positive effect on the body-weight of adrenalectomized rats (fig. 2 and 3), but it was clearly less active in this respect than 11-desoxycorticosterone acetate.

17 (β)-hydroxy-progesterone was administered to rats, which were made to work immediately following adrenalectomy, in doses of 1, 2, and 3 mg. twice daily with negative results. The compound 6-hydroxy-progesterone acetate was similarly tested in amounts up to 2 mg. per dose with negative results.

It has not been possible to make thorough quantitative studies of the biologic properties of the compounds reported here with the exception of progesterone. However, it can be definitely concluded that none of these compounds are as active in their effect on work as corticosterone and similar adrenal steroids which are oxygenated at carbon 11, and none of these compounds are as active in maintaining the body weight of adrenalectomized rats as 11-desoxycorticosterone acetate. In view of the negative effect of Δ^4-17a-methyl-D-homo-androstene-17a-ol-3, 17-dione on body-weight and the fact that it does not belong to the corticosterone series, the claim by Ruzicka and Meldahl (3) that it is similar to corticosterone in its life maintenance activity is open to question.

SUMMARY

Several steroid compounds were examined for possible effects upon the body-weight and work performance of adrenalectomized rats. Large doses of progesterone enabled the animals to gain in weight and to perform more work than untreated animals but the work performance remained very inferior to that of normal animals. Progesterone, 17 (β) hydroxy-progesterone, and 6-hydroxy-progesterone acetate all failed to enhance the work performance of adrenalectomized rats when the test was conducted immediately following operation. The compound Δ^4-17a-methyl-D-homo-androstene-17a-ol-3, 17-dione was without a positive effect on either body-weight or work performance. The compound 6-hydroxy-11-desoxycorticosterone diacetate did not enhance the work performance of rats tested immediately following adrenalectomy but in large dosage it enabled rats to gain in weight following adrenalectomy. Although more active than progesterone it was definitely less active per mg. in this respect than 11-desoxycorticosterone acetate.

I wish to express my appreciation to Professor L. Ruzicka, Zürich, Switzerland, who supplied the Δ^4-17a-methyl-D-homo-androstene-17a-ol-3, 17-dione; to Dr. E. Schwenk of the Schering Corporation, Bloomfield, New Jersey, who supplied the progesterone and the 11-desoxycorticosterone acetate; to Dr. J. J. Pfiffner of Parke, Davis and Company who supplied the 17-hydroxy progesterone; and to Dr. M. Ehrenstein of this laboratory who supplied the 6-hydroxy-progesterone acetate and the 6-hydroxy-11-desoxycorticosterone diacetate.

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INHIBITION OF THE CARMINE-CELL REACTION IN THE PITUITARIES OF CATS WHICH MATE BUT DO NOT OVULATE

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RABBITS NORMALLY OVULATE 10 to 12 hours post coitum; ovulation does not ordinarily occur spontaneously in this species. It has been demonstrated that post-coital ovulation can be prevented *a*), if the infundibular stalk is severed prior to mating; *b*), if the pituitary gland is removed within 60 to 90 minutes after mating. Hypophysectomy does not interfere with ovulation if it is done later than 90 minutes post coitum (1–7). These observations indicate that the behavioral phenomena which are characteristic of coitus induce ovulation in the rabbit by virtue of their stimulating effect upon the gonadotropic activity of the anterior hypophysis.

Cytological evidence of this pituitary stimulation by coitus has been reported by Friedgood and Dawson (8, 9). In the anterior pituitary of the rabbit they found a hitherto unrecognized cell characterized by large, coarse, irregular granules with a marked affinity for azocarmine. These cells, present in moderate numbers in the pituitary of the estrous rabbit, are significantly increased in number within one-half hour after mating. This increase in number could be detected macroscopically in many instances because of their aggregation into compact, irregularly circumscribed areas.

Friedgood and Dawson (10, 11) subsequently demonstrated similar cells in the pituitary of the cat, an animal which, like the rabbit, usually ovulates only post coitum. Although morphologically different from those described in the rabbit pituitary, these cells had an identical affinity for azocarmine. Studies of the functional significance of the carmine cells revealed that they were not present in the pituitary of the anestrous cat. They first appear in a limited area of the anterior pituitary during proestrus, and then increase significantly in numbers and extent of distribution during estrus. After mating they are arranged in a characteristic alveolar pattern, and their greatly increased numbers and extent of distribution reach a maximum within 5 to 6 hours. Degranulation, which occurs as early as 4 hours post coitum, becomes more pronounced after 6 hours; and the carmine reaction is virtually over at 14 hours.

The precise functional significance of these carmine cells is unknown. Correlation of physiological and cytological data suggest that in the postcoital period they are related to the secretion of the luteinizing hormone (LH) of the pituitary. It is our present view, however, that the carmine cells may be associated in some way with other gonadotropic functions of the anterior pituitary gland, because they are present in large numbers in the pituitary of the cat in the last week of pregnancy and during early lactation.

In further experiments it was found that coitus, which normally induces ovulation in most cats, does not do so if the animals are subjected to various operative proce-

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dures shortly after mating, such as a second adrenalectomy (one adrenal having been removed several weeks previously); this prevents ovulation if the operation is performed within 15 to 55 minutes post coitum; but ovulation does occur if the operation is delayed for 6 hours (12). Anesthesia alone, given for a period comparable to that of the average duration of an operation, does not interfere with the occurrence of ovulation. In subsequent experiments, it was determined (13) that absence of the adrenals is not the specific cause of the failure of ovulation; exploratory laparotomy and a number of other non-specific abdominal operations were found to have a similar inhibitory effect. The observations indicated that abdominal surgery, done within one hour after mating, interferes in some way with the usual response of the anterior pituitary to coital stimulation. The present observations were made to determine the extent of distribution and character of the carmine-cell reaction in the pituitaries of the foregoing series of cats, and the results of these studies were correlated with a coincident histological study of the follicles in the ovaries.

RESULTS

The pituitaries and ovaries of 10 mated and operated cats were not examined histologically until after an interval of 46 hours or more had elapsed without the occurrence of ovulation since the latter occurs normally about 26 hours post coitum. A similar study was made of 2 other estrous but unoperated cats (*1M*, *2M*, table 1) which did not ovulate after mating and after artificial stimulation of the cervix uteri, respectively. The carmine cells in the pituitaries of 8 of the first group of 10 animals (*1MI* through *8MI*) were comparable in number and distribution to those in estrous cats. The pituitary cells of 7 of these 8 cats showed no evidence of degranulation and their ovarian follicles were normal. In the 8th animal, (*8MI*) which was autopsied 47 hours post coitum, degranulation of the carmine cells was almost complete, and the ovarian follicles, somewhat comparable to those of a normal unoperated cat about 10 hours post coitum (14), showed an atypical cumulus and first maturation spindles in 3 follicles of one ovary. This may have been an activation of atretic follicles since the follicles were smaller than other typical estrous follicles which were present in the same ovary; on the other hand, suboptimal stimulation may best explain the atypical character of the follicular reaction. The foregoing evidence indicates that degranulation of pituitary cells is coincident with secretion of the gonadotropic substances, and in this instance the carmine cells, although completely degranulated, were limited in number to that characteristic of the estrous level. In contrast to the other 8 cats in this group, the pituitaries of the remaining 2 animals showed an extensive postcoital carmine-cell reaction, but there was very little degranulation in one (*9MI*) and none in the other (*10MI*). Only estrous follicles were found in the ovaries of these 2 animals. The pituitary of a mated but operated cat (*1M*) which did not ovulate showed mild evidence of the carmine-cell reaction, and its ovaries contained only normal estrous follicles. The pituitary of an unoperated estrous cat (*2M*) whose cervix uteri was artificially stimulated with a glass rod, showed a normal postcopulatory carmine-cell reaction at 29 hours except for the absence of degranulation. The ovarian follicles of this animal were likewise of the characteristic estrous variety.

DISCUSSION

In those cats which failed to ovulate after mating because of laparotomy, the extent of the carmine-cell reaction in the pituitary was comparable to that of the average estrous animal. In certain circumstances there was also some modification of the granulation of the carmine cells in the pituitaries of those animals as compared with normally mated but unoperated cats. The carmine cells of animals autopsied

TABLE 1. PITUITARIES OF CATS WHICH MATED BUT DID NOT OVULATE

Cat	Experimental conditions	Date of autopsy	Hours after mating and/or stimulation	Extent and distribution of carmine-cell reaction and granulation	Condition of ovarian follicles and ova	Sexual behavior before, during and after mating
1MI	Ether started 20 min. after mating. Blank laparotomy	7/26/37	120	Estrous extent; few carmine cells	Estrous follicles	Typical estrous behavior with treading and screaming during coition, 7/21/37; did not execute characteristic postcoital tossing and rolling movements after licking vaginal orifice. When autopsied 5 days later, (7/26/37) was not in heat although the follicles appeared to be of the estrous variety.
2MI	Ether started 25 min. after mating. Left nephrectomy	4/27/38	168	Estrous extent; few carmine cells	Follicles appear to be regressing	Mated 3 times; artificial stimulation twice. Typical estrous behavior during and after both procedures. Not in heat before autopsy.
3MI	Ether started 25 min. after mating. Left nephrectomy	2/25/38	67.3	Estrous extent; few carmine cells	3 large estrous follicles in left ovary. 1 large follicle in rt. ovary.	Mated twice; artificial stimulation twice. Typical estrous behavior during and after both procedures.
4MI	Ether started 17 min. after mating. Left nephrectomy	6/28/37	72	Estrous extent; few carmine cells	Estrous follicles	Mated 3 times; artificial stimulation once. Typical estrous behavior during and after both procedures.
5MI	Ether started 25 min. after mating. Left nephrectomy	4/18/38	46	Estrous extent; some areas with little granulation; other areas fully granulated.	Estrous follicles	Mated twice; artificial stimulation 4 times. Although mated eagerly, did not scream during coitus and the postcoital movements were moderate.
6MI	Ether started 25 min. after mating. Partial splenectomy	3/24/38	46	Estrous extent; granulated	4 large estrous follicles	Mated twice; artificial stimulation once. Typical estrous behavior during and after both procedures.

TABLE 1. PITUITARIES OF CATS WHICH MATED BUT DID NOT OVULATE

Cat	Experimental conditions	Date of autopsy	Hours after mating and/or stimulation	Extent and distribution of carmine-cell reaction and granulation	Condition of ovarian follicles and ova	Sexual behavior before, during and after mating.
7MI	Ether started 25 min. after mating. Partial splenectomy	5/14/38	48	Estrous extent; complete granulation	Estrous follicles	Mated 3 times eagerly with typical estrous behavior.
8MI	Ether started 25 min. after mating. Partial splenectomy	3/24/38	47.8	Estrous extent; degranulation almost complete.	Atypical cumulus reaction; 1st maturation spindle in 3 follicles of one ovary; activation of atretic follicle?	Mated once; artificial stimulation once. Typical estrous behavior during and after both procedures.
9MI	Ether started 20 min. after mating. Coeliac ganglionectomy	6/30/37	48	Postcopulatory response; little degranulation.	Cumulus vacuolated as in late estrus.	Mated eagerly 3 times with typical estrous behavior.
10MI	Left nephrectomy 38 min. after coitus	7/16/37	192	Postcopulatory response; normal granulation.	Estrous follicles. Compact cumulus, few large vacuoles	Artificial stimulation 4 times, 7/8/37. Response characteristic of coital and postcoital estrous behavior. Similar stimulation, 7/12/37, elicited same response. On 7/13/37 cat was found in heat again and laparotomy revealed the presence of estrous-appearing follicles. On 7/16/37 the animal was still in heat just before autopsy.
1M	No anesthesia or surgery	2/15/39	49.3	Estrous extent; slight degranulation	Estrous follicles, compact cumulus	Moderate treading when mounted; moderate cry during coitus. No postcoital movements except for slight licking of vaginal orifice. Mated once; artificial stimulation 3 times.
2M	No anesthesia or surgery	5/24/41	29	Postcopulatory response; normal granulation.	Estrous follicles with vacuolated cumulus	Artificial stimulation 4 times with good response.

about 48 hours after coitus or artificial stimulation usually possessed a normal amount of granules. The granulated carmine cells in the pituitaries of animals autopsied later in the postcoital period (72 to 168 hours after mating) were reduced in number, but the original extent of the carmine reaction could be determined from the regional loss of granules. This delayed loss of granules probably represents regression of the carmine cells rather than active release of their secretion, because there is no unequivocal evidence of preovulatory activation of the mature, ovarian follicles in any of these cases.

The carmine-cell reaction of the pituitaries of 2 other cats (*9MI* and *10MI*) was extensive enough to equal that usually found after successful copulation; but significant degranulation did not occur in either instance and there was no evidence of preovulatory follicular activation. It is important to recall in this connection that a marked carmine-cell reaction is not exclusively a postcopulatory phenomenon. It occurs in an occasional unmated, estrous cat which exhibits maximal estrous behavior (11).

The pituitary glands of cats *1M* and *2M* were strikingly different, although both were removed from normal estrous-behaving animals. In *1M* the coital stimulation was evidently insufficient to initiate a carmine reaction beyond the estrous level. This may have been due to the degree of estrus since the ovarian follicles possessed compact cumuli characteristic of late proestrus rather than of typical estrus. On the other hand, the cumuli of the ovarian follicles of *2M* were highly vacuolated and typical of estrus (14). The carmine reaction in this animal was comparable to that following a successful mating, but no degranulation was evident at 29 hours.

The condition of all the large ovarian follicles of the 12 cats in this study were remarkable in that none of them, with the possible exception of *8MI* and *2MI* showed any evidence of atresia or any change in the follicular cells other than those normally found in estrous follicles. Furthermore, mitotic activity was an outstanding feature of the cells of the cumulus and granulosa of all the follicles studied.

On the basis of these and previously published observations (8–11) the following generalizations may be made.

Various non-specific abdominal operations in the cat interfere with the usual effect of coitus which normally stimulates the gonadotropic activity of the anterior pituitary gland and thus induces ovulation. The extent of the carmine-cell reaction in the pituitaries of such cats which mate but do not ovulate is usually significantly less than that which is found in animals after a normal mating. Consequently, the characteristic postcoital increase in the number and extent of distribution of the carmine cells can be directly correlated with an activation of that function of the anterior pituitary which results in ovulation. The carmine-cell reaction in these animals, therefore, constitutes histological evidence of an enhanced gonadotropic activity of the pituitary gland. The extent of the carmine-cell reaction alone cannot be correlated, however, with the secretion of the gonadotropic substance which actually induces ovulation. Presumably, therefore, the postcoital carmine-cell reaction is an anatomic expression of the physiologic processes which are concerned with the elaboration of the gonadotropin or its change from the state in which it is stored to an active secretory form.

The occurrence and amount of degranulation is apparently the determining factor in the maturation of follicular ova and ovulation. Lack of degranulation of an extensive carmine-cell reaction leaves the ovarian follicles in an estrous state; partial degranulation may or may not be associated with minimal evidence of maturation depending on the original extent of the carmine-cell reaction; massive degranulation of a widespread

carmine-cell reaction appears to be essential for completion of maturation and subsequent ovulation.

It may be concluded, therefore, that *a*) coitus stimulates an increase in the number of carmine cells by virtue of which the gonadotropic activity of the anterior pituitary is enhanced; *b*) the carmine cell contains a hormone necessary for the initial maturation of the follicles and subsequent ovulation; and *c*) degranulation represents the secretory phase during which, if the extent of the carmine-cell reaction is sufficient and the degranulation adequate, enough hormone is released to initiate maturation and induce ovulation.

This interpretation accounts satisfactorily for the fact that no interference with ovulation was observed in animals operated upon 6 hours post coitum. At this time the carmine reaction has passed its maximum, there is widespread evidence of degranulation, and the cumulus reaction in the ovarian follicle has already begun. The former is probably indicative of the release of hormone from the carmine cells, while the latter is early evidence of the ovarian effects of this hormone. The operation apparently comes too late in the chain of physiological events, therefore, to alter significantly the normal course of ovarian stimulation by the pituitary.

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EFFECT OF THE GONADOTROPIC SUBSTANCE OF PREGNANT MARE'S SERUM ON THE BLOOD PLASMA-ASCORBIC ACID OF THE BOVINE

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PRIOR TO 1939, it was generally agreed, as summarized by Mason (1), that only man, monkey and the guinea pig required extraneous sources of ascorbic acid; that vitamin C deficiencies in these species probably caused no specific reproductive disturbances; and that the high ascorbic acid content of such endocrine glands as the adrenal cortex, corpus luteum, anterior pituitary, ovary and testis was not directly related to reproductive or endocrine functions.

However, evidence has been accumulating during the past decade which indicates that vitamin C may play a part in the regulation of normal reproductive processes and that the utilization of ascorbic acid may be partially under endocrine control. Biskind and Glick (2) found that the corpus luteum of pregnancy in the guinea pig contained twice as much vitamin C as that of the non-pregnant female, and Ley (3) reported that ascorbic acid was of therapeutic value in the treatment of habitual abortion in women. Phillips et al. (4, 5) have found that subcutaneous injections of ascorbic acid have resulted in the restoration of fertility in certain impotent bulls and that similar treatment has been of value in the treatment of some types of sterility in the cow.

Mosonyi (6) reported that the injection of either the male or female sex hormones resulted in a reduction of the ascorbic acid content of the liver and adrenals; Winkler and Binder (7) found that prolan decreased the amount of vitamin C present in the pituitary; and Israel and Meranze (8) have recently reported that ascorbic acid has a progesterone-like effect on the endometrium of the ovariectomized mouse, rat and rabbit.

During the routine determination of the blood plasma-ascorbic acid of a group of bulls it was found that there was a great reduction in the amount of vitamin C present after the injection of equine gonadotropic hormone. This study was conducted to determine whether or not the gonadotropic hormone has a rôle in the regulation of venous blood plasma-ascorbic acid in the bovine.

MATERIALS AND METHODS

Venous blood plasma-ascorbic acid determinations were made by the slightly modified method of Taylor et al. (9). Blood samples of approximately 15 cc. were drawn into oxalated centrifuge tubes and taken to the laboratory for immediate analysis. Duplicate 2 cc. samples of plasma were pipetted directly into 8 cc. of 6 per cent meta phosphoric acid, stirred vigorously and the precipitated protein removed by centrifugation. Five cc. aliquots of the supernatant liquid were titrated

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[1] The gonadotropin (Gonadin) used was the product of the Cutter Laboratories, Berkeley, California.

with Tillman's reagent and the reduced ascorbic acid determined. Tillman's reagent was prepared by dissolving 20 mg. of 2, 6 dichlorophenol indophenol in phosphate buffer at a pH of 6.8.

The gonadotropic hormone[1] used in these experiments was purchased on the open market, and is described by the producers as equine pregnancy serum standardized to contain 50 R.U. per cc.

Two bulls and 4 cows were injected subcutaneously with amounts of gonadotropic hormone varying from 1000 to 2250 R.U. during 8 individual trials. In certain experiments to determine the effect of extraneous vitamin C upon the blood plasma-ascorbic acid following gonadotropin injection, crystalline vitamin C was administered subcutaneously in sterile saline solution. The individual trials and procedures will be described under results.

The diets of all of the animals remained constant throughout the trials and to the best knowledge of the investigators, variations in food intake of vitamin C did not influence the data recorded.

RESULTS

Trial 1, 3-year-old *Guernsey bull*. This animal was given 5 injections of 3 gm. of ascorbic acid each during the period between April 14 and May 1, 1941, but received no injected vitamin C during the 26 days prior to or following the injection of 2250 R.U. of gonadotropin on May 27, 1941. As shown in figure 1 and table 1 there was

TABLE 1. EFFECT OF EQUINE GONADOTROPIC HORMONE ON BLOOD PLASMA-ASCORBIC ACID

Animal	Trial No.	Gonadotropin	Blood Plasma-Ascorbic Acid			
			Day of injection	Hours following injection		
				9	24	48
		R.U.	mg. %	mg. %	mg. %	mg. %
Guernsey bull	1	2250	0.60	0.33	0.20	0.22
Guernsey bull	2	2250	0.60	0.30	0.24	0.21
Guernsey bull	3	2250	0.62	0.46	0.36	0.43
Ayrshire bull	4	2250	0.42	0.21	0.24	0.39
Guernsey cow 452	5	1500	0.54	0.43	0.36	0.36
Guernsey cow 540	6	2250	0.58	0.38	0.29	0.32
Holstein cow 90A	7	1000	0.64	0.53	0.51	0.41
Holstein cow 73A	8	1200	0.62	0.47	0.46	0.37

a precipitous drop in venous plasma-ascorbic acid during the 24-hour period following administration of the hormone. The plasma content of reduced ascorbic acid decreased 45 per cent during the first 9 hours and 67 per cent during the first 24 hours.

Trial 2 was made on same *Guernsey bull*. Three days before the injection of 2250 R.U. of gonadotropin 6 gm. of ascorbic acid were administered subcutaneously and the plasma level of vitamin C had risen to 0.60 mg. per cent on the day of gonadotropin injection. During the 9-hour period following treatment the plasma-ascorbic acid decreased 50 per cent and at the end of 24 hours had fallen 60 per cent (table 1). No additional vitamin C was administered and the plasma ascorbic acid had risen to 0.33 mg. per cent 10 days following gonadotropin injection.

Trial 3 was also made on same *Guernsey bull*. This experiment was conducted for the purpose of determining whether or not continued daily injections of ascorbic acid would decrease the fall of plasma-ascorbic acid following gonadotropin injection and would hasten the return of plasma-vitamin C to preinjection levels. As shown

in figure 1, 2 gm. of ascorbic acid were administered daily during the period 15 days prior to and 15 days following gonadotropin treatment. The plasma-ascorbic acid leveled off at approximately 0.60 mg. per cent 10 days prior to the injection of 2250 R.U. of gonadotropic hormone, dropped 26 per cent during the first 9 postinjection hours and 42 per cent during the first 24 hours. Fifteen days after gonadotropin treatment the blood-ascorbic acid had risen to pre-injection levels. Although the same dosage of gonadotropic hormone was administered it is apparent that the decrease in plasma-ascorbic acid was at least partially inhibited by continued vitamin C injection.

Trial 4, 4-year-old *Ayrshire bull.* This animal was given no ascorbic acid injections prior to gonadotropin treatment. During the 9-hour period following injec-

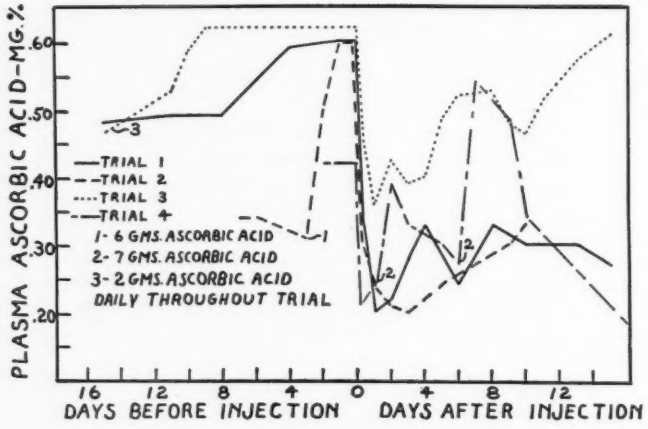

Fig. 1. Effect of equine gonadotropin upon venous blood plasma-ascorbic acid of the bull.

tion the plasma-ascorbic acid decreased 50 per cent and had risen slightly 24 hours later. On the 1st and 6th days following treatment 7 gm. of ascorbic acid were injected. As shown in figure 1 there was an immediate but temporary rise in plasma-ascorbic acid and at the end of 16 days the level was lower than at any previous time.

Trial 5, 4-year-old *Guernsey cow* 452. This cow was nonpregnant and lactating. She had not exhibited estrus for more than 15 months, indicating, at least, that estrogenic substances were not being produced in appreciable amounts. Following the injection of 1500 R.U. of gonadotropin, blood plasma-ascorbic acid dropped 20 per cent during the first 9 hours and 32 per cent at the end of 24 hours. The vitamin C value of the blood had risen to approximately pre-injection levels at the end of 11 days. Estrus was not induced by the gonadotropic hormone.

Trial 6, 3-year-old *Guernsey cow* 540. This animal was nonpregnant, lactating and apparently normal. During the first 9 hours following the injection of 2250 R.U. of gonadotropin, plasma-ascorbic acid decreased 36 per cent and had decreased 50 per cent at the end of 24 hours. Recovery to approximate pre-injection levels occurred in 7 days and estrus was not induced by treatment.

Trial 7, 1-year-old *Holstein heifer* 90A. This animal was nonpregnant, nonlactating and apparently normal. The plasma-ascorbic acid fell gradually following the injection of 1000 R.U. of gonadotropin and the maximum decrease of 36 per cent

was observed on the 2nd day. Nearly normal values were observed on the 10th postinjection day. This female received the smallest dose of gonadotropic hormone, exhibited the least response, and estrus was not induced.

Trial 8, 2-year-old nonpregnant, lactating *Holstein cow* 73A. This animal failed to exhibit estrus at the expected time 2 days prior to the injection of 1200 R.U. of gonadotropin but showed pronounced estrus from the 4th to 7th postinjection days inclusively. The maximum decrease of plasma-ascorbic acid was observed on the

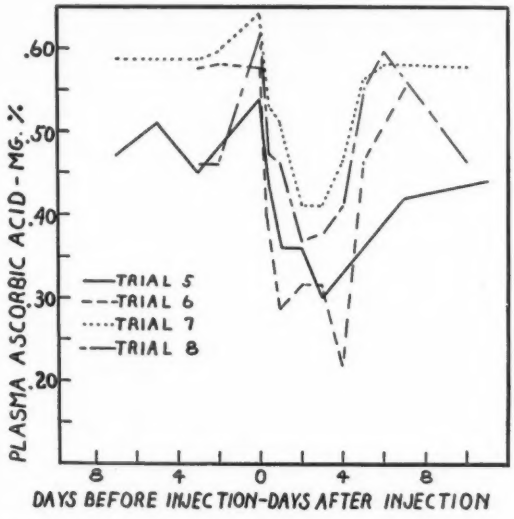

Fig. 2. EFFECT OF EQUINE GONADOTROPIN UPON VENOUS BLOOD PLASMA-ASCORBIC ACID OF THE COW.

2nd day, but the level rose markedly during estrus (fig. 2). Following the termination of estrus there was a pronounced drop in blood plasma-ascorbic acid, a phenomenon described previously by Phillips (5).

DISCUSSION

Although individual differences in venous blood plasma-ascorbic acid of cattle maintained under similar conditions are often marked, and although unexplained daily variations in vitamin C blood levels occur, the consistent decreases in plasma-ascorbic acid which were observed following the injection of equine gonadotropic hormone indicate a relationship between these substances in the bovine. The findings of Phillips et al. (5) that the average diestrous levels of plasma-ascorbic acid were 0.364 mg. per cent and the average estrous levels 0.475 mg. per cent lend support to the hypothesis that venous blood plasma-ascorbic acid is at least partially under endocrine control. The mechanisms involved remain obscure, and the drop in plasma-vitamin C may be due to increased usage of the vitamin by all or particular body tissues, to body storage, to excretion in the urine or feces, or to combinations of these. Studies on the bull indicate that the return to normal plasma-ascorbic acid levels proceeds slowly unless additional vitamin C is injected and that in the cow, with the dosages of gonadotropin used, recovery was approached in about 10 days.

Although various gonadotropic substances have been used with success in in-

ducing ovulation and spermatogenesis in certain species, clinical experience has often been disappointing. If ascorbic acid is concerned with normal reproductive processes and if injected gonadotropic hormones cause a reduction in blood plasma-ascorbic acid, the possibility exists that the decrease in vitamin C might be sufficient to interfere with the physiological processes which the gonadotropic hormones should theoretically induce. It would seem important, therefore, that additional studies be carried out on the hormonal regulation of ascorbic acid metabolism.

SUMMARY

The injection of 2250 R.U. of gonadotropin into the dairy bull caused a decrease in venous blood plasma-ascorbic acid of 42 to 67 per cent during the first 24-hour period.

The injection of 1000 to 2250 R.U. of hormone into the cow caused decreases of 20 to 50 per cent during the first 24-hour period.

The recovery of ascorbic acid to approximate pre-injection levels required 8 to 10 days in the cow and longer in the bull unless extraneous vitamin C was injected.

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THE INFLUENCE OF ASCORBIC ACID ON THE ACTIVITY OF GONADOTROPIC HORMONES

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IT IS RECOGNIZED THAT deficiency of vitamin supply may lead to disturbed functions of some glands of internal secretion, and that addition of the missing vitamin to the diet may correct the condition. Numerous experimental studies have been reported, bearing on the influence exerted by vitamin C on the development and function of the gonads. Favorable effects have been seen in both sexes.

In this connection special interest attaches to the observations of Giedosz who simultaneously injected gonadotropic hormone and ascorbic acid for 3 days into adult

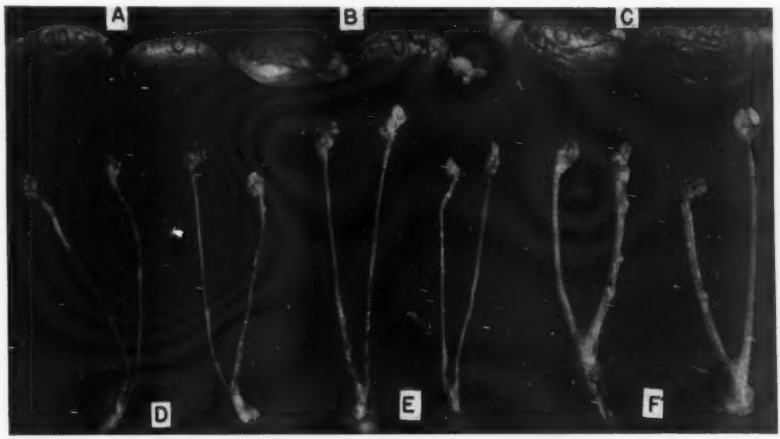

Fig. 1. *A*, TESTIS, NORMAL RAT. *B*, testis, rat injected with gonadotropic hormone. *C*, testis, rat injected with gonadotropic hormone and ascorbic acid. *D*, reproductive tract of normal female rat. *E*, reproductive tract of female rat injected with gonadotropic hormones. *F*, reproductive tract of female rat injected with gonadotropic hormones and ascorbic acid.

rabbit does, producing larger ovaries than in animals receiving the hormone only. The author concludes that the efficiency of these hormones is greater if the organism is saturated with vitamin C. Giedosz administered 1,050 mg. of ascorbic acid in 3 days.

We have studied the favorable influence of vitamin C not only during the short periods as done by Giedosz, but also for longer times, 20 to 30 days. As test animals we used white rats, 2 to 4 months old. Daily injections of 50 mg. of ascorbic acid[1] and 25 R.U. of gonadotropic hormone[2] were given. We employed 4 groups of 12 animals each.

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[1] Redoxon, Roche.

[2] Menstruina, Lea.

From each group 4 animals were injected with gonadotropic hormone and ascorbic acid simultaneously, 4 others with hormone only, and 4 with vitamin only. The results were compared with 4 groups of uninjected, normal rats. The effects on female gonads are shown in figure 1 D, E and F, with technique comparable to that of Giedosz. The development of the testes is shown in figure 1 A, B and C and in the table. The weights are the average for the two testicles in the respective animals. The average penis weight was twice as great in those animals treated with both gonadotropic hormone and ascorbic acid, as in those given hormone only.

The female genital organs showed similar greater growth from the combined hormone and vitamin.

Ascorbic acid alone produced no definite effect on testicular size, as shown by the

TABLE 1. EFFECT OF GONADOTROPIC HORMONE AND ASCORBIC ACID ALONE AND COMBINED ON TESTES OF RATS

	Wt. of testicles in gm.		
Ascorbic acid and gonadotropic hormone	Gonadotropic hormone	Ascorbic acid	Normals
1.50	0.725	0.320	0.340
0.945	0.450	0.275	0.270
1.07	0.750	0.502	0.448
1.08	0.765	0.410	0.385
Av. 1.15	0.672	0.377	0.361

table when comparing results in the 3rd and 4th columns. In 3 other male rats injected with ascorbic acid daily for 15 days, testis weight was 1.75, 1.85, and 1.10 gm. In 3 rats fed a bread and milk diet, without ascorbic acid, testis weight was 1.70, 1.90, 1.05 gm., showing no significant differences. The results of injecting ascorbic acid into adult rabbits following the technic of Giedosz showed no variations in favor of the animals given both vitamin and hormone.

We deduce from our investigation that simultaneous injection of gonadotropic hormone and ascorbic acid for 20 to 30 days produces a greater increase in development of testis and penis, and of female genitalia than the hormone alone. The increase is almost doubled by the combined treatment as compared with the hormone alone. Ascorbic acid alone produced no change in gonad size in normal rats. In short periods of treatment (3 days) and in adult animals the effect of the vitamin seems of little importance.

We are applying these observations to clinical problems, and expect to publish the results in due time.

STEROID METABOLISM: ESTROGENS AND PHENOLASES[1]

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IN A PREVIOUS PAPER (1) we reported that mushroom laccase oxidized estrogens in a 20 to 26.6% alcohol solution, whereas both mushroom and potato tyrosinase failed to do so. We employed alcoholic solutions because of the relative insolubility in water of estradiol, estrone, and estriol. The ineffectiveness of the tyrosinases used might be attributable to their great sensitivity to alcohol (2), although control experiments with catechol in alcoholic solution showed no marked inactivation of the tyrosinase. The estrogenic substrates are, however, oxidized much more slowly than catechol. This difference in oxidation rate, and the presence of an induction period, might allow for an alcohol effect upon the tyrosinases sufficient to block a possible oxidation. Accordingly Dr. William H. Pearlman prepared for us 4 completely water-soluble estrogen derivatives: estriol succinate, estrone carboxymethoxime, estradiol succinate and estradiol phthalate.[3] All of these could be dissolved stoichometrically by 0.01 M NaOH and brought to pH 7.0. Since these are esterified through the auxiliary OH groups of ring D, the phenolic portion of the molecule may be considered 'free.'

METHODS AND MATERIALS

In the sidearm of a Barcroft-Warburg respirometer vessel was placed 0.5 cc. of the hormone ester solution containing 2 mg./cc. In the main chamber was the enzyme dissolved in respective phosphate buffer (2.3 cc.); in the central well of the main chamber was 0.2 cc. 15% KOH. Enzyme and hormone solutions were ordinarily mixed after a short equilibration period in a thermostat at 27°C. Experiments with animal tissue preparations were conducted at 37°C.

Potato tyrosinase was prepared from grated, washed potatoes. The potato dough was pressed out thoroughly through muslin and the liquid obtained was centrifuged to remove starch. The supernatant fluid treated with 0.4 saturated $(NH_4)_2SO_4$ was allowed to stand overnight. The precipitate formed was removed by centrifugation and the supernatant solution adjusted to 0.8 saturation with $(NH_4)_2SO_4$. The precipitate formed was collected by centrifugation, dissolved in distilled water and dialyzed. The dialysate contained the enzyme. Mushroom tyrosinase (from *Psalliota campestris*) was prepared in the same way. Mushroom laccase was prepared as previously described (3).

RESULTS

Potato tyrosinase. In figure 1 is presented typical data on the oxidation of 3 estrogen esters by potato tyrosinase at pH 7.2. It should be noted that the reaction velocities are nearly identical, but that the duration of the induction period varies with the

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[1] Aided by a grant from the National Research Council Committee for Problems of Sex. Work Projects Administration Project No. 65-1-14-2949.
[2] Finney-Howell Research Fellow.
[3] The estradiol was supplied through the kindness of Dr. Erwin Schwenk of the Schering Co. Bloomfield, N. J.

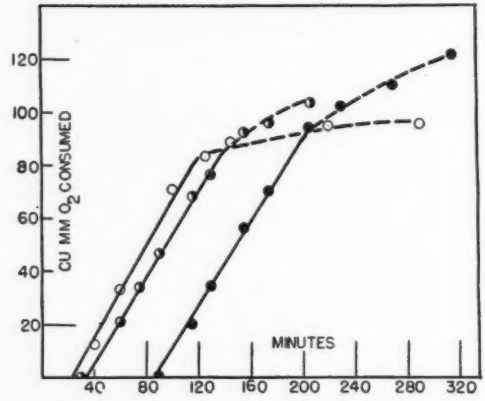

Fig. 1. Oxidation of 3 water-soluble estrogen esters by potato tyrosinase; pH 7.3; temperature 27° C. ●—estradiol phthalate (1 mg.); ◑—estrone carboxymethoxime (1 mg.); ○—estradiol succinate (1 mg.).

type of substrate acted upon by a given enzyme preparation. The data of this and a number of similar experiments indicate an oxygen uptake of 3 to 4 atoms per molecule of substrate. The end products were all reddish in color.

The data of figure 2 demonstrate that the usual enzyme-substrate relationship holds for the estrogenic phenol, that is, that the oxidation rate was proportional to the enzyme concentration. The usual inhibitors of tyrosinase activity (KCN and salicylaldoxime) inhibited the oxidation of the estrogens. As is demonstrated in figure 3, the presence of estrone carboxymethoxime brought about the complete oxidation of ascorbic acid which is normally not a substrate for tyrosinase. This is an effect typically occurring with other phenol-tyrosinase combinations (4, 3). In the presence of aniline at an acid pH phenols form aniliniquinone products (5). A similar precipitate

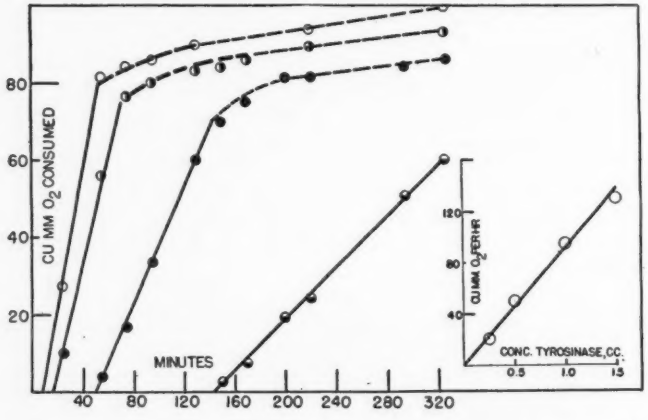

Fig. 2. Effect of various concentrations of potato tyrosinase on rate of oxidation of estrone carboxymethoxime. The amount of estrogen was in each case 1 mg.; pH 7.3; temperature 27° C. ○—1.50 cc. tyrosinase; ◑—1.00 cc. tyrosinase; ●—0.50 cc. tyrosinase; ◒—0.25 cc. tyrosinase. *Inset*: the concentration of tyrosinase plotted against the rate of oxygen uptake.

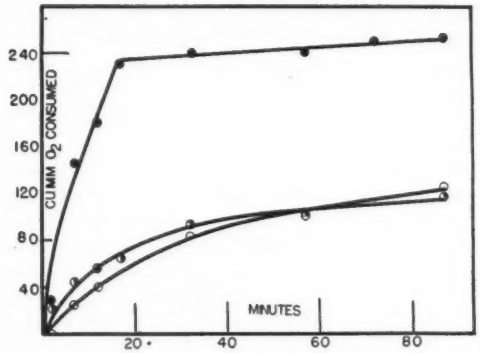

Fig. 3. Effect of ascorbic acid and aniline upon the oxidation of estrone carboxymethoxime by potato tyrosinase at acid pH. The amount of estrogen was in each case 1 mg.; pH 6.0; temperature 27° C. ○—estrogen and tyrosinase; ●—estrogen, tyrosinase, and ascorbic acid (2 mg.); ◑—estrogen, tyrosinase and aniline (5 mg.).

was formed with the estrogenic substrates (fig. 3). Typically the induction period in tyrosinase oxidation was prolonged by oxidizing substances and eliminated by reducing substances (6). Ascorbic acid and aniline abolished the induction period in estrogen oxidation (fig. 3), cytochrome prolonged it (fig. 4). The cytochrome effect was not due simply to added protein since in other experiments there was no effect of added gelatin on the induction period.

It was found that potato tyrosinase oxidation was not confined to the estrogenic steroidal phenols. Stilbestrol may be rendered water-soluble by lecithin. Using a ratio of 10 parts of lecithin to 1 part of stilbestrol thoroughly mixed in ether solution, the

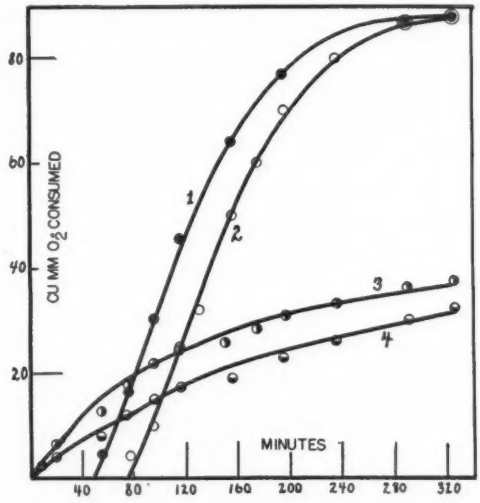

Fig. 4. A comparison of the oxidation of estrone carboxymethoxime by potato tyrosinase and mushroom laccase. The amount of estrogen was in each case 1 mg.; pH 7.3; temperature 27° C ●—estrogen and potato tyrosinase; ○—estrogen, potato tyrosinase and cytochrome; ◑—estrogen and mushroom laccase; ◒—estrogen, mushroom laccase and potato tyrosinase.

precipitate formed after evaporating the ether could be taken up in water (or buffer solution) in a concentration of 11 mg. per cc. A high oxygen uptake was obtained, but due to the fact that lecithin itself is oxidized in this system it was difficult to obtain exact quantitative figures on the degree of oxidation. We should expect an oxygen uptake of about 8 atoms per molecule of stilbestrol since it is a dihydroxyphenol. This result is approximated.

Mushroom tyrosinase. In no instance were we able to obtain any appreciable oxygen uptake when the estrogenic phenols were added to mushroom tyrosinase solutions. Highly active mushroom tyrosinase preparations have been employed in various concentrations over the effective pH range of the enzyme. The addition of small quantities of possible oxygen carriers such as catechol and p-cresol did not effect oxidation of the estrogens. The estrogens did not appear to act as poisons to the tyrosinase since other phenolic substrates were readily oxidized in the presence of the estrogens. This peculiar behavior of the mushroom tyrosinase toward the estrogens may be accountable to a prolonged induction period but the addition of ascorbic acid to the system failed to cause any hastened oxidation.

TABLE 1. EFFECT OF VARIOUS PHENOLASES ON THE ESTROGENIC ACTIVITY OF CERTAIN ESTROGENS

Enzyme	Substrate	% of original activity recovered
Potato tyrosinase	estradiol succinate	0.0[1]
Potato tyrosinase	estradiol phthalate	0.0[2]
Potato tyrosinase	estrone carboxymethoxime	0.0[2]
Mushroom tyrosinase	estriol succinate	100.0
Mushroom tyrosinase plus ascorbic acid	estriol succinate	100.0
Mushroom laccase	estradiol succinate	4.0
Mushroom laccase plus potato tyrosinase	estrone carboxymethoxime	10.0
Cytochrome oxidase of beef heart	estradiol succinate	90.0

[1] Tested for 1/300 of original activity.
[2] Tested for 1/100 of original activity.

Mushroom laccase. The water-soluble estrogen esters were more readily oxidized by laccase than the free hormones in alcoholic solution in the sense that the oxygen uptake was the same for each of the estrogenic substrates. Previously we found that the oxygen uptake in alcoholic solution seemed to vary with the solubility of the individual estrogens (1). The total oxygen uptake was ¼ to ⅓ of that obtained with potato tyrosinase (compare *curves* 1 and 3, fig. 4), and amounted to approximately one atom of oxygen per molecule of substrate. The reaction product was not colored.

In the presence of tyrosinase, laccase tended to dominate the reaction (*curve* 4, fig. 4). The rate of oxidation was like that obtained with laccase and the end products were colorless.

Tissue oxidases. Attempts to obtain oxidases from mammalian tissue were instituted particularly in view of the reported inactivation of estrogens by the liver (7, 8, 9). In numerous experiments with washed and unwashed rabbit liver extracts with and without added cytochrome, no appreciable oxygen uptake occurred on the addition of the water-soluble estrogen esters. Furthermore, preparations of cytochrome oxidase from various sources (uterus, heart, muscle) failed to oxidize any of the estrogen esters.

Estrogenic activity of the phenolase products. Assays of the products of reaction of the three phenolases were conducted upon spayed rats by the method of Smith and Smith and Schiller (10). The results are detailed in table 1. They demonstrate that the inactivation by potato tyrosinase was probably complete whereas mushroom tyro-

sinase was ineffective even in the presence of ascorbic acid. This is consonant with the oxidizing activity of the two preparations. Laccase, on the other hand, inactivated 90% to 96% of the hormone esters. Again, this parallels the oxidative behavior since the oxygen uptake of laccase preparations was usually equivalent to a little less than one atom of oxygen per molecule; in other words, about 5 to 10% of the substrate was not oxidized. The data on the laccase-potato tyrosinase mixture indicate, as do the oxygen uptake data (fig. 4) that the laccase dominated the reaction. The mild inactivation found after incubating estradiol succinate with cytochrome oxidase was probably a nonspecific effect.

DISCUSSION

The behavior of the phenolases towards the estrogenic phenols parallels their activity toward phenols generally but differs markedly in one respect. Thus laccase shows a lower oxygen uptake than potato tyrosinase and produces a colorless end product. This is exactly the behavior of the two enzymes with p-cresol. But the ineffectiveness of mushroom tyrosinase both as an oxidase and as an estrogen inactivator is unique. Hitherto no distinction between tyrosinase from the two sources has been possible. Westerfeld (11) has previously reported the inactivation of microgram quantities of estrogen by mushroom tyrosinase. It may be that his preparations contained sufficient admixture of laccase to affect the quantities of estrogen he employed. Our mushroom tyrosinase preparations were free of laccase.

The ineffectiveness of cytochrome oxidase is not surprising in view of the fact that it does not attack monohydroxyphenols generally. This implies that if the estrogens are oxidized enzymically in the organism as phenols they are first converted to dihydroxyphenols; alternatively we may assign the oxidation to tissue enzymes other than cytochrome oxidase. If such enzymes are phenolases we have not found them by ordinary methods; perhaps a protyrosinase plus its activator is necessary (12). Our inability to obtain an oxidase particularly from liver may imply that the liver inactivates estrogen by a detoxification (esterification) mechanism rather than an oxidation.

SUMMARY

With preparations of water-soluble esters of the native estrogens (estriol, estrone and estradiol) experiments with oxidases for the phenolic ring are made possible. Potato tyrosinase oxidizes the estrogens to form colored end products with the uptake of 3 to 4 atoms of oxygen per molecule of hormone. The hormones appear to be completely inactivated. Tyrosinase obtained from the common edible mushroom does not appreciably oxidize the estrogens, nor does it affect their estrogenic activity. Laccase oxidizes the estrogens with an uptake of about one atom of oxygen per molecule of hormone and with a loss of 90% of the hormone activity. Neither cytochrome oxidase nor various tissue preparations give any evidence of estrogen oxidation activity. Beef heart cytochrome oxidase does not appreciably affect the estrogenic activity of the soluble estrogen esters.

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STEROID EXCRETION IN CANCEROUS AND NONCANCEROUS PERSONS[1]

I. THE 17-KETOSTEROIDS

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THIS STUDY appeared to be fruitful in view of the close structural similarity of carcinogenic phenanthrene hydrocarbons and steroids and the rôle which estrogens and androgens play in the incidence of spontaneous mammary carcinoma in mice (1). Differences in 17-ketosteroid excretion had already been noted by Werthessen and Pincus (2). In this paper a report is made on the 17-ketosteroids in pooled urines of males who had carcinoma of the gut and bladder and of females who suffered from carcinoma of the breast and uterus. No known or suspected cases of malignancy of the adrenal cortex were included. The urines from noncancerous persons included those of healthy and also of hospitalized individuals with no apparent endocrine disorder. Attention was focused on the digitonin-precipitable hydroxyketones since reports had been made of a markedly increased excretion of this fraction in malignancies of the adrenal cortex (3–6); comparative studies of this fraction could also be made with a greater relative degree of accuracy. A separate report on estrogen studies is given elsewhere (7).

The ketones obtained from ether extracts of acid-hydrolyzed urines were separated into alcoholic and nonalcoholic fractions. The digitonin-precipitable hydroxyketones were purified over the semicarbazones. Table 1 gives the data for the weights of the various fractions and the respective 17-ketosteroid titers on a liter basis. The latter were determined colorimetrically by a modified Zimmerman reaction (8). The digitonin-precipitable hydroxyketones were not thus estimated. Instead, the weights of the purified fractions are given since only 17-ketosteroids were isolated from these in good yield; the semicarbazones are but slightly soluble, so that little loss in 17-ketosteroid material may be expected.

The data indicate that noncancerous persons of both sexes have a higher total 17-ketosteroid output and a markedly higher output of digitonin-precipitable hydroxyketones than cancerous persons. Noncancerous males excrete roughly twice as much 17-ketosteroid compounds as do noncancerous females; this ratio holds for all of the fractions listed. On the other hand, cancerous males and females tend to excrete approximately the same amount of neutral 17-ketosteroids. This is equally true for all of the fractions listed.

The digitonin-precipitable hydroxyketones.[2] Isoandrosterone was obtained as the sole product from the urine of noncancerous females and cancerous males and females. Dehydroisoandrosterone could not be isolated. Urine from noncancerous males yielded an apparently homogeneous crystalline product (m.p. 156.5–157.5°C. $[\alpha]_D + 50°$).

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[1] Aided by grants from the Dazian Foundation for Medical Research and G. D. Searle & Company. Work Projects Administration Project No. 65-1-14-2949.

[2] A preliminary report on this fraction has been given (9).

This appears to be a mixture, probably of dehydroisoandrosterone and isoandrosterone, as indicated by the carbon and hydrogen values and the specific optical rotation. A similar product was readily obtained by crystallizing combined equal parts of the authentic steroids. Practice chromatography indicated very poor separation of the constituents of the mixture. The Schoenheimer method for separating unsaturated from saturated digitonin-precipitable alcohols (10) was unsatisfactory when applied to the small quantity of alcoholic ketones that were available.

The data for the digitonin-precipitable hydroxyketonic fractions of normal urines are at variance with previous reports in the literature. Callow and Callow (11) ob-

TABLE 1.[1] WEIGHTS OF NEUTRAL FRACTIONS AND THE 17-KETOSTEROID TITERS,[2] EXPRESSED IN MG. PER LITER

Source of urine	Total neutral fraction	Total ketonic fraction	Alcoholic ketonic fraction	Digitonin-ppt.[3] alcoholic ketones	Nonalcoholic ketonic fraction	Non ketonic
Noncancerous males (188 liters)	44.2 (14.70)	18.6 (11.62)	9.41 (5.73)	0.52 —	6.97 (5.51)	23.8 —
Cancerous males (476 liters)	24.0 (3.90)	7.56 (3.68)	3.55 (1.65)	0.08 —	3.34 (1.73)	15.5 —
Noncancerous females (146 liters)	25.1 (5.69)	9.45 (5.38)	5.89 (2.66)	0.25 —	2.40 (2.00)	13.0 —
Cancerous females (231 liters)	28.9 (3.99)	11.7[4] (3.58)	4.59 (1.74)	0.09 —	2.60 (1.52)	18.4 —

[1] Discrepancies in values are ascribed to losses incurred by the chemical manipulations in fractionating; there is approximately a 5–10% loss in the ketonic separation and a 10–15% loss in the alcohol separation.

[2] The 17-ketosteroid values are in parenthesis.

[3] Based on the weight of the hydrolysates obtained from the crystalline semicarbazones.

[4] This fraction was not completely dry when weighed; the 17-ketosteroid value is, however, not affected.

tained dehydroisoandrosterone from normal female urine (incidentally, urine from hospitalized women was included). The failure to isolate this steroid may perhaps be ascribed to the vigorous hydrolytic procedure employed in this laboratory since it has been clearly demonstrated that dehydroisoandrosterone is sensitive to boiling hydrochloric acid and that great losses of this steroid may thereby occur (8). On the other hand, the British investigators failed to report the presence of isoandrosterone. Dehydroisoandrosterone has been reported by others (12–15) as the sole digitonin-precipitable hydroxyketone from urines of normal males and females, although of these investigators, Butenandt considered the presence of isoandrosterone in normal male urine not unlikely. A seemingly plausible explanation for these discrepancies is that isoandrosterone arises as an artefact from bacterial hydrogenation of dehydroisoandrosterone in urine. However, such a supposition is inconsistent. If bacterial hydrogenation had occurred, one could hardly expect the rather close check obtained for the two batches of urine from noncancerous males and at the same time obtain markedly different results for the urines of noncancerous males and females (Callow's data for urines of males and females are similar both qualitatively and quantitatively). Since the question of bacterial hydrogenation is a critical one, the author attempted to ascertain the effects of urinary putrefaction on dehydroisoandrosterone. An aqueous solu-

tion of the succinate derivative was prepared and added to urine, which was then permitted to stand for 11 days without toluene at room temperature; dehydroisoandrosterone was isolated as the sole product in high yield. It is, of course, possible that the conjugated form of this steroid as excreted in the urine may be more labile in this respect. The fractionation procedure adopted in this laboratory may be the factor responsible for the discrepancies. Callow chromatographed the entire ketonic fraction and may have lost track of isoandrosterone which is present in minor proportions. Engel (15) treated the hydroxyketonic fraction of urine from normal males with digitonin. This procedure was independently adopted in this laboratory but the subsequent fractionation differs.

The singular results obtained by the author necessitate further investigation of the digitonin-precipitable hydroxyketones of human urines before arriving at unequivocal conclusions. The presence of isoandrosterone is not in itself surprising from a biogenetic point of view. Dorfman (16) isolated abnormal amounts of isoandrosterone after administering testosterone propionate to a hypogonadal male. Callow (14) failed to isolate this steroid in a similar experiment. Both investigators used essentially similar techniques of isolation. An origin from the adrenal is also indicated since large amounts of isoandrosterone were isolated by Butler and Marrian (4) from unhydrolyzed urine in a case of adrenal tumor. It is interesting that no dehydroisoandrosterone was found, whereas in a study of an adrenal tumor by Wolfe et al. (5) abnormally high yields of dehydroisoandrosterone but no isoandrosterone were obtained.

The low excretion of total 17-ketosteroids, especially the digitonin-precipitable fraction in patients with cancer is significant in connection with a current theory of the adrenal origin of these steroids. The inference is that the adrenal cortex is functioning at a low level. Whether age, malignancy, or some other factor is responsible for this effect is not clear at the moment but it is hoped that studies of individual cases now in progress will shed light on this point.

The nonalcoholic ketones. Androstenone-17, m.p. 104–105°C. was obtained from the urines of noncancerous females. This steroid has been recently reported by Hirschmann (16) as a constituent of the urine of ovariectomized women. It had not been previously isolated from the urine of normal women. A similar product was obtained by Engel (15) from the urine of normal males. Urine from cancerous males is not lacking in this steroid. According to Hirschmann, androstenone-17 may be Δ^2-androstenone-17 or Δ^3-androstenone-17 or a mixture of both. This probably accounts for the variety of melting-points (102, 107.5–110, 111–114.5, 110 and 111°C.) reported for this product depending on the mode of preparation. This author observed slight discrepancies in the melting-point data for the urinary and synthetic androstenone-17 preparations, as well as for the respective oxime derivatives.

The androstenone-17 which was isolated by this author gave a faintly positive Rosenheim reaction whereas the crude fractions from which these were derived gave intense red colors in this test. Synthetic androstenone-17 gives a negative Rosenheim reaction. The finding that the specific optical rotation of the crude crystalline fractions increased and approached the value for synthetic androstenone-17 $[\alpha]_D+151°$ (17) on recrystallization suggested contamination with a compound such as $\Delta^{3,5}$ androstadienone-17. The latter steroid is levorotatory and gives a positive Rosenheim reaction. In the case of the urines of noncancerous males, a crystalline mixture, m.p. 92–96°C., $[\alpha]_D+83°$ was obtained. It gave an intensely positive Rosenheim reaction. Attempts at further purification failed.

Experiments conducted on a micro-scale, involving hydrochloric acid treatment of dehydroisoandrosterone under conditions simulating that of urinary hydrolysis, revealed the presence of Rosenheim positive material in the nonalcoholic fraction (8).

An experiment conducted on a semi-micro scale failed to yield any crystalline material in this fraction. 3-chloro-Δ^5-androstenone-17, which is conceded to be an artefact, does not give the Rosenheim reaction, not even when subjected to succinic anhydride-pyridine treatment. Burrows et al. (3) could not obtain the doubly unsaturated steroid from dehydroisoandrosterone after hydrochloric acid treatment. Wolfe et al. (5) are of the opinion that $\Delta^{3,5}$ androstadienone-17 is an artefact but that it does not arise from dehydroisoandrosterone. It should be pointed out that such model experiments have been made on unesterified dehydroisoandrosterone and not with the conjugated form in which it is actually excreted and whose constitution is as yet unknown. It is not inconceivable that the latter form may be more labile in this respect.

TABLE 2. SOURCES OF URINES CONTRIBUTED TO URINE POOLS

Urine pool	No. liters	Types of contributors	Age range yr.
14 Noncancerous males, I	188	2/3 from non-hospitalized, normal men. 1/3 from hospitalized men with peptic ulcer, prostatitis, Hodgkins disease, leg amputation.	26–55 42–68
Noncancerous males, II	276	All from non-hospitalized, normal men	16–58
10 Noncancerous females	146	3/4 from non-hospitalized, normal women. 1/4 from patients with ovarian cyst, non-malignant cervical erosion, neurosis.	18–63
31 Cancerous males	476	Malignancies of stomach and intestines (32%) bladder (26%) prostate (22%) throat (10%) mouth (4%) lungs (3%) pancreas (2%) skin (1%).	30–83
33 Cancerous females	231	Malignancies of cervix (30%) stomach and intestines (26%) breast (17%) ear (15%) lungs (7%) bladder (3%) antrum (2%).	32–80

EXPERIMENTAL PROCEDURE[3]

Selection of patients. Table 2 indicates the percentage contribution of nonhospitalized individuals to the noncancerous pools, the types of malignancies and also the age range for the various groups.

Hydrolysis and fractionation procedures. The urinary specimens were collected and hydrolyzed within 3 or 4 days. Toluene was used as a preservative. To 10 volumes of urine, 1.5 volumes of concentrated hydrochloric acid were added, the mixture rapidly brought to a boil and the boiling continued for 7 minutes. Extraction was made with ether. Acidic, phenolic and neutral fractions were obtained by usual procedures. The procedure of Girard and Sandulesco (18) was employed in order to obtain the ketonic material from the neutral extracts.

The separation of alcoholic from nonalcoholic ketones was accomplished by a procedure essentially that of Dutcher and Wintersteiner (19). Half-esterification with succinic anhydride in pyridine was carried out at room temperature for 16 hours and finally by heating for one hour at 90–100°C. A second treatment with succinic anhydride yielded an additional 10 per cent of hydroxyketonic material. The hydroxyketonic fractions were treated with 2 per cent digitonin in 70 per cent methanol. The insoluble digitonides were dissolved in dry pyridine, dry ether added to precipitate the digitonin, and then worked up. The free digitonin-precipitable hydroxyketones

[3] All melting points reported are corrected. The microanalyses were made by Mr. William Saschek (College of Physicians and Surgeons, New York).

were refluxed for 2 hours with semicarbazide acetate in 90 per cent alcohol. The crystalline semicarbazones were hydrolyzed by refluxing for 2½ hours with 0.5 N hydrochloric acid in 80 per cent alcohol.

The Digitonin-Precipitable Hydroxyketones

Urine I, from noncancerous males. An amount of 99 mg. of crystalline hydrolysate, obtained from 137 mg. of crystalline semicarbazone, dec. 271°, was recrystallized twice from absolute methanol to yield 49 mg. of rod-like crystals, melting at 156.5–157.5°C. The melting-point was not changed by further recrystallization.

$[\alpha]_D^{20°} = +50°$, 1.09 per cent in ethanol.

Analysis:	$C_{19}H_{28}O_2$	calculated C 79.11, H 9.79
	$C_{19}H_{30}O_2$	calculated C 78.57, H 10.41
		found C 78.83, H 9.88

An additional 29 mg., m.p. 151–153°C. was obtained from the mother liquors.

Seven mg. of the product melting at 156.5–157.5°C. were benzoylated in pyridine at room temperature. Six mg. of a crystalline product were obtained, m.p. 237–239°C. The melting-point was not affected by further recrystallization.

Ten mg., m.p. 156.5–157.5°C., were refluxed in acetic anhydride for 1½ hours. Six mg. of crystalline material, melting at 147–157°C., were obtained. Two recrystallizations from the same solvent gave 0.5 mg., m.p. 160–161.5°C. A mixed melting-point with dehydroisoandrosterone acetate (m.p., 168–169°C.) was 162–164°C.

Urine II, from noncancerous males (non-hospitalized) (260 liters). Eighty-four mg. of white crystalline hydrolysate $[\alpha]_D^{23°} +49°$, 2.10 per cent in ethanol, were obtained from 106 mg. of crystalline semicarbazone, dec. 264–265°. It was recrystallized twice from methanol to yield 55 mg., m.p. 156.5–157.5°C. and $[\alpha]_D^{29°} +45°$, 1.28 per cent in ethanol.

When 34.2 mg. of isoandrosterone, m.p. 170–171°C., $[\alpha]_D^{29°} +88°$ (0.95 per cent in ethanol) and 34.2 mg. of dehydroisoandrosterone, m.p. 147–148°C., $[\alpha]_D^{29°} +12°$ (1.92 per cent in ethanol), were combined and crystallized once from methanol, 49.6 mg. of apparently homogeneous crystals were obtained. The melting-point was 157.5–158.5°C. and $[\alpha]_D^{29°} +51°$ (1.45 per cent in ethanol). It gave no mixed melting-point depression with the urinary product, m.p. 156.5–157.5°C.

Urine from cancerous males. Twenty-four mg., m.p. 158–160°C., were obtained from 39 mg. of semicarbazone hydrolyzate by crystallization from absolute methanol. Two recrystallizations from the same solvent yielded 10 mg., m.p. 165°C., which did not depress the melting-point of isoandrosterone, m.p. 170–171°C. The product was benzoylated in the usual manner. Five mg., m.p. 215–217°C., were obtained by several recrystallizations from ethanol; its mixed melting-point with isoandrosterone benzoate (m.p. 219–220°C.) was 218–219°C.

Analysis:	$C_{26}H_{34}O_3$	calculated C 79.14, H 8.69
		found C 78.80, H 8.55

Urine from noncancerous females. Thirty mg., m.p. 165°C., were obtained from 36 mg. of semicarbazone hydrolyzate by crystallization from methanol. Two recrystallizations from the same solvent and a high vacuum distillation ($10^{-4} - 10^{-5}$ mm. Hg) yielded 15 mg. of crystalline sublimate ($[\alpha]_D^{+20°} = +78$, 0.37 per cent in ethanol). Recrystallization from methanol yielded 11 mg., melting at 168–169°C.; a mixed melting-point with isoandrosterone (m.p. 170–171°C.) was 169–171°C. An additional 10 mg., m.p. 162–164°C. were obtained from the mother liquors. Benzoylation and two recrystallizations from ethanol yielded 9 mg., m.p. 213–215°C. A mixed melting-point with isoandrosterone benzoate was 213–217°C.

Urines from cancerous females. Twenty mg. of semicrystalline material, obtained

from the hydrolysis of the crystalline semicarbazone, were sublimed in a high vacuum at 110–125°C. and yielded 18.5 mg. of a white crystalline sublimate. Two recrystallizations from methanol gave 5 mg., m.p. 161–163°C. Four mg. of this product were benzoylated. Recrystallization from ethanol yielded 3 mg., m.p. 210–211°C., which did not depress the melting point of authentic isoandrosterone benzoate.

Non-Alcoholic Ketones

Urine from noncancerous males. One and thirty-one hundredths gm. of the non-alcoholic ketonic material were chromatographed from a benzene-pentane mixture over aluminum oxide (Brockmann). Elutions with benzene-pentane mixtures, benzene, and acetone were made. The benzene-pentane eluates were refluxed with semicarbazide acetate; the crystalline semicarbazones which were obtained were refluxed for two and one-half hours with 0.5 N hydrochloric acid in 80 per cent alcohol. The hydrolyzate consisted of semicrystalline material (157 mg. 17-ketosteroid equivalents according to the Zimmerman reaction with dehydroisoandrosterone as standard. It was then chromatographed over aluminum oxide. A benzene-pentane eluate yielded 42 mg. of crystalline material ($[\alpha]_D^{25°} = +75°$, 1.05 per cent in ethanol). Recrystallization from methanol yielded 21 mg. of rectangular plates, which did not melt sharply at 92–96°C. ($[\alpha]_D^{25°} = +83°$, 0.51 per cent in ethanol). This product gave an intense red color when a few crystals were dissolved in 90 per cent trichloracetic acid (Rosenheim test).

Urines from cancerous males. Four hundred seventy-five mg. of a benzene-pentane eluate were obtained by a similar chromatographic analysis of 1.59 gm. of nonalcoholic ketonic material. It was distilled in a high vacuum at 75–140°C. Three hundred fifty mg. of a light yellow oil were obtained which on treatment with semicarbazide acetate yielded 68 mg. of crystalline semicarbazone, dec. 280–283°. Hydrolysis in the usual manner followed by chromatographic analysis yielded 20 mg. (benzene-pentane eluate) of crystalline material ($[\alpha]_D^{24°} = +103°$, 0.5 per cent in ethanol). Recrystallization from methanol gave 10 mg. of rectangular plates, melting at 104–105°C. ($[\alpha]_D^{24°} = +138°$, 0.24 per cent in ethanol). 5 mg., m.p. 107.5–108.5°C. were obtained on further recrystallization. It gave a faint red color in the Rosenheim test. A mixed melting-point determination with synthetic androstenone-17 (m.p. 104–105°C.) prepared according to the method of Hirschmann (17) was 106–107°C.

The oxime derivatives were prepared by refluxing for two hours in 90 per cent alcohol containing an excess of hydroxylamine acetate. The crystalline precipitates which were obtained by the addition of water were filtered, washed repeatedly with water and then recrystallized from aqueous alcohol. Four mg. of synthetic androstenone-17, m.p. 104–105°C. yielded 3 mg. of oxime, m.p. 148–149°C. while 6 mg. of the urinary product, m.p. 106.5–108.5°C., yielded 5 mg., m.p. 153–154°C. The melting-point of a mixture of these oximes was 152–153°C.

Urine from noncancerous females. One hundred sixty-one mg. of a benzene-pentane eluate, obtained by chromatographing 353 mg. of nonalcoholic ketonic material, yielded 44 mg. of crystalline semicarbazone dec. 283–284°. Hydrolysis, followed by chromatographic analysis, yielded 24 mg. of crystalline material in a benzene-pentane fraction; $[\alpha]_D^{25°} +108°$, 0.61 per cent in ethanol. Recrystallization from methanol yielded 15 mg. of rectangular plates, m.p. 101–102°C., $[\alpha]_D^{25°} +119°$, 0.38 per cent in ethanol. Two more recrystallizations from the same solvent yielded 7 mg., m.p. 104–105°C. It gave a faintly positive Rosenheim test. A mixed melting-point with synthetic androstenone-17 was 104–105°C. Five mg. of the oxime derivative, melting at 149–150°C. were obtained by treatment in the manner indicated above. A mixed melting-point determination with the synthetic product was 149–150°C.

Urine from cancerous females. Similar treatment yielded only 18 mg. of a crude amorphous semicarbazone preparation. It was not submitted to further examination.

SUMMARY

Cancerous persons excrete significantly less 17-ketosteroids than do noncancerous persons. This effect is particularly noticeable in the digitonin-precipitable hydroxyketonic fraction. The malignancy may not be the direct causative factor.

Noncancerous males excrete significantly greater amounts of 17-ketosteroids than do noncancerous females. This sex difference appears to be abolished in cancerous persons.

Isoandrosterone is a normal constituent of human urines. It is also present but in much smaller quantities as the sole constituent of the digitonin-precipitable hydroxyketonic fraction in pooled urines from cancerous persons. The question of artefact formation is discussed.

Dehydroisoandrosterone failed to be isolated from urines of noncancerous females. The presence of this steroid and isoandrosterone in urine of noncancerous males is indicated.

Androstenone-17 was isolated from urines of noncancerous females and cancerous males.

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THE CHICK COMB FOR ANDROGEN ASSAY[1]

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THE RELIABILITY of the chick comb test for androgen assay has been seriously questioned recently. A series of preliminary papers by Frank and his coworkers (1–5) suggested several methods for the elimination of some of the variables which modify the comb growth response and with improved technique the use of the chick comb promised to be successful. Three papers, however, from different laboratories (6, 7, 8) presented data which seemed contrary to those of Frank et al. and evoked a reply from the latter workers (9) in which it was stated that these experiments were not comparable nor sufficiently critical.

In the course of our studies with White Leghorn chicks it has been noted that the comb of the cockerel is often very responsive to small amounts of androgen, notably dihydroandrosterone benzoate and testosterone propionate. We have also observed (10) that the results of androgen injection during inanition are especially marked and that such animals appear to be excellent for assay purposes. Consequently, a number of experiments were planned to determine whether or not the responses of limited-diet animals (inanition) were critical enough for assay purposes. Because of the controversial nature of the question the data have been withheld until a large number of birds (1318 to date) could be studied.

The chick test should possess several attributes in order to be advantageous and to improve upon the present test which uses comb growth in capons for male hormone assay: *a*) small amounts of androgen must give positive responses, preferably with less than 50 μg. of male hormone; *b*) a positive response should be evident without resort to extensive mathematical analysis because the average biologist is not well enough acquainted with advanced statistical methods to make elaborate calculations for each assay; and *c*), the cost should be less than for the capons. If a moderately sized series of from 12 to 20 animals could be used for each assay level, the cost would be reasonable.

MATERIALS AND METHODS

Single comb White Leghorn chicks were used in all the experiments. These were secured from one hatchery within 12 hours of hatching. They were sexed at the time of hatching and were shipped in the usual 'chick pullman.' It has been my good fortune to secure birds which were carefully sexed and it has been possible to depend on at least a 95% accuracy which frequently has been better than 99 per cent. Testosterone propionate in sesame oil was used in all experiments but one and injections

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were made with a 1 cc. tuberculin syringe graduated into 1/100 cc. The amount of oil used in subcutaneous injections was 0.1 cc. and in the comb 0.02 cc. The chicks were reared in a battery brooder and contrary to the suggestion of Frank et al., controls and androgen-treated birds were kept together. Inanition was controlled by removing the food on alternate days during the first 8 or 10 days of age and then restricting the feeding time even more after the 10th day by limiting the feeding to only a few hours on the days when food was given.

Only the best grade of chicks which were blood tested and which were healthy when received were used in our work. Care was taken that they did not chill at any time and food and water was carefully checked at all times. The birds were watched so that none was tramped upon or caught in the mesh floor of the cage. With these precautions it is unusual to encounter more than 2% mortality and during the rigorous diet restriction there is seldom more than an 8% mortality. The experiments which are designated as the '80-hour test' were similar to those reported by Byerly et al. (11). Animals were injected and kept in the chick pullman without food or water for 80 hours, after which they were autopsied. This has been a successful test for small amounts of gonadotropin (11, 12) and was accordingly tried as a test for androgens. A viability of 100% is common in such series. The baby capons were castrated at 5 days of age and the technique used was similar to that employed for older fowls, namely entry between the last two ribs, but the removal of the testes was done with jeweler's forceps. The incision was on the left side and the operation was performed under a binocular microscope with a light attached to the nose piece. Ether anesthesia was used. Contrary to a rather common belief, this is a successful means of anesthetizing baby chicks. There have been only two fatalities which could be blamed directly on the anesthetic during several hundred operations in these and other experiments.

A preliminary injection of androgen was given in all of the experiments reported in this paper with one exception. This preliminary injection has been referred to as a 'priming dose' and was 5 or 10 μg. of testosterone propionate in 0.1 cc. of sesame oil given subcutaneously in a single injection into the neck two days before the experimental dosages were administered. Control chicks were given the priming dose and also received sesame oil daily. Comb sizes were first determined as a percentage of body weight and the percentage increases, shown in the graphs, represent the increment produced by the injections when the birds injected with the priming dose plus sesame oil were used as controls.

RESULTS AND DISCUSSIONS

Normal-diet animals proved to be less responsive to androgens than did the limited-diet chicks in previous work (10). One important factor in this reaction is the fact that the chick testes are secreting some comb-growth-promoting substance (13) and that the androgen which is injected acts through the pituitary gland to suppress testis growth and to some extent testis secretion (14). This is very well demonstrated by the data which are shown in figure 1.

The injection of 25 and 50 micrograms of the male hormone caused some suppression of comb growth. The probable explanation is that there was a diminished secretion of testicular androgen resulting indirectly from an inhibition of the anterior pituitary secretion. This more than offset the stimulation produced on the comb by the injected male hormone. Support for this idea is found in the fact that testis weights in the injected series were only 46 to 62 per cent of the control weights. It was suggested earlier (10) that a correction factor might be applied in such cases by calculating the comb data on the basis of a uniform testis size. This would assume that the

testicular hormone secreted is exactly proportional to the amount of testis tissue and although this is doubtful, such a mathematical treatment serves to correct within certain limits for decreased testis hormone output. When the correction for a uniform testis weight in all series was made (10 mg.) the variation from the normal comb size produced by 25 μg. and 50 μg. dosages was not significant, but the response to 100 μg. was shown to be relatively much greater. This material was not studied extensively because, as will become apparent, the limited-diet chicks proved much more responsive.

Figure 2, *A* and *B* demonstrates the results of preliminary experiments using the chicks which were on a reduced food intake. Limitation of diet has been shown to de-

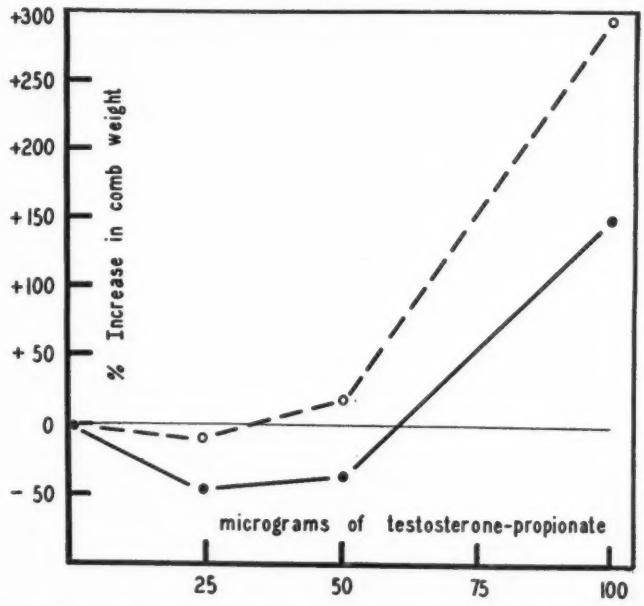


Fig. 1. SIXTY ANIMALS, 15-day-normal-diet cockerels which received 10 μg. of testosterone propionate on 2nd day as a priming dose and were injected daily from the 4th to 13th with the same preparation. Dotted line is the comb response as corrected for a uniform testis weight average of 10 mg.

crease greatly both pituitary and gonad secretion (12, 13) and for this reason the animals approach a condition of pseudohypophysectomy and the necessity for making corrections in comb sizes for uniform gonad weights can be eliminated except perhaps at high dosage levels. The data for the pullets shown in the graph demonstrate clearly that at dosages from 10 to 1000 μg. the comb will respond readily to the androgen. There is some evidence of a plateau in the curve when the 500 microgram dosage is reached but it is not my intention at this time to discuss the possible factors involved in the lowered response at this level.

The average comb size at the 10 μg. hormone level was 26 per cent greater than the control for both the male and female limited-diet chicks but above this there was some divergence of results. These series were not all run at the same time but several months elapsed between experiments; for this reason a strict comparison should not

be made. The pullets show in general a greater response except at the 10 and 100 μg. dosages but the form of the curves is similar which is significant. The response of the limited-diet females did not exceed that of the males in later work in which both sexes were treated at the same time; for that reason in subsequent graphs in which the cockerels and pullets were injected the two groups were averaged together after the percentage increases of each group had been calculated. It is unnecessary, therefore, to use a correction factor for the females because, as was indicated, the dieted animals

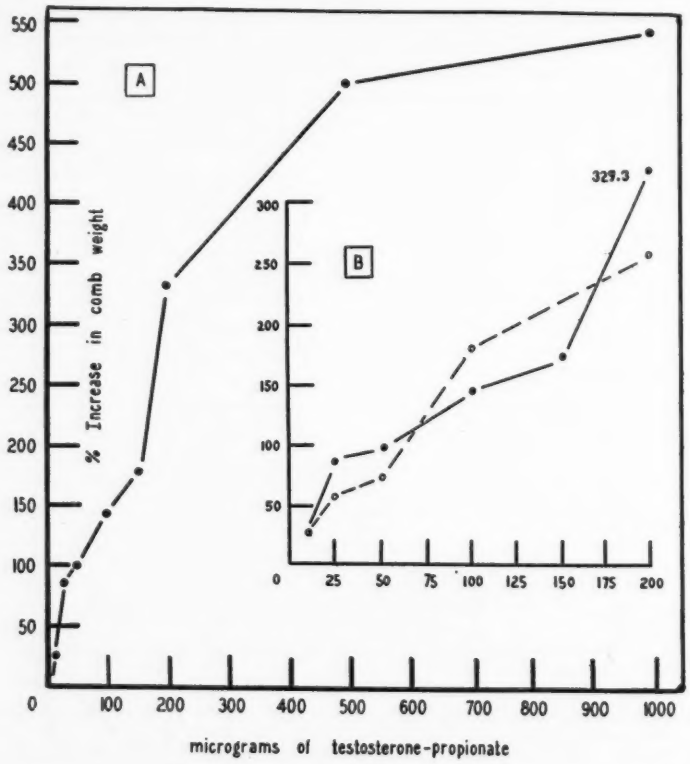


Fig. 2, A. Two hundred and 71 animals, 15-day-limited-diet females given 10 μg. of testosterone propionate as a priming dose on 2nd day with subsequent daily injections from the 4th to 13th day of the same preparation. *B.* 15-day-limited-diet males shown by dotted line plotted with the females over a shorter dosage range, 133 males.

are essentially physiological castrates. The device of employing a factor for normal-diet females should be seriously questioned, however, on the basis of past experience.

It was shown in a recent paper (13) that the comb of the male increases from .0453 per cent of body weight on the 10th day of age to .1090 per cent on the 15th day. The pullet comb during the same period grows only from .0256 per cent to .0280 per cent of body weight. At the 20th day after hatching the relative comb sizes are .1640% for the cockerels and .0417 per cent for the females. The relative size at 20 days in the females is, therefore, only approximately the equivalent of that in the ten-day-old male. It is difficult to find any single factor which will express the relationship for the

comb in the two sexes for different days during this period from the 10th to 20th day during which the cockerel comb has increased about 3.6 times in relative size but the pullet comb only about 1.7 times. This divergence in size continues to increase and at the 30th day the male comb exceeds the female comb in proportion to body weight by 1000 per cent.

It is certain that those who employ comb factors for correction of data from mixed sex groups of experimental normal-diet birds must use different factors for the various age groups. Failure to recognize these physiological variations between the two sexes in the hormonal control of comb increment will lead to serious errors and undoubtedly account for the fact that some laboratories cannot agree at present on what 'comb factor' to employ.

One series of limited-diet chicks was given free testosterone instead of the propionic ester and these results are presented in figure 3. These data demonstrate that

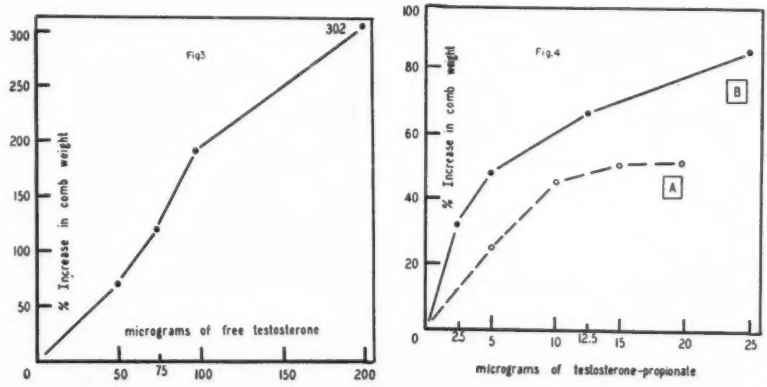


Fig. 3. SIXTY ANIMALS, 15-day-limited-diet pullets given 10 μg. of testosterone propionate as a priming dose on second day followed by daily injections of testosterone from the 4th to 13th day. Fig. 4, A. TWO HUNDRED AND 48 ANIMALS, 15-day-limited-diet males and females given 5 μg. of testosterone propionate as a priming dose on 2nd day with subsequent daily injections into the comb of the same androgen from the 4th to 13th day. B. 141 birds, 15-day-limited-diet cockerels and pullet given 5 μg. of testosterone propionate as priming dose on second day followed by injections of the same preparation into the comb on days 4, 6, 8, 10, 12. All comb injections were in 0.02 cc. of sesame oil.

significant responses in the comb may be produced by the free hormone, and, indeed, the comb growth even exceeds the response to testosterone propionate at the 100 μg. dosage. The injection of 50 μg. of the free testosterone produced an increment of 70.3 per cent in the comb which was significant and indicates that even with free testosterone satisfactory analysis may be made at and probably below this dosage.

It was noted during autopsy of the animals that all the oil had not been absorbed and in a few birds which were permitted to go beyond the termination of the regular experimental period, combs continued to show marked growth with evidence of continued action of injected androgen. This fact suggested that the response, although demonstrating the effect of the hormone, did not represent the full measure of the androgen present. Further modification of the technique was advisable. There were two possibilities immediately applicable: *a*) to change the site of the injections; *b*) to give the same amount of hormone but in fewer injections and in a smaller total amount of sesame oil. Some of the investigations referred to in the introductory statements in this paper have employed the direct application of hormone to the comb. It was thought that perhaps the latter method might be practical and, therefore, comb injec-

tions were made and smaller dosages of the male hormone administered. These data are presented graphically in figure 4.

The curve *A* in figure 4 represents the percentage increase produced by the daily injection of testosterone propionate in 0.02 cc. amounts into the comb and represents a total of 248 chicks of both sexes in almost equal numbers. At the 5 and 10 μg. dosage levels, the response is much greater than that shown in figure 2, in which the injections had been made subcutaneously. The method is, therefore, valuable in the determination of still smaller amounts of androgen than in the previous experiments. However, there is a very definite plateau in the response following the injection of 15 and 20 μg. of the hormone and it appeared likely that the comb was unable to respond fully to such dosages. The possible explanation for this difficulty was offered by the presence of considerable quantities of oil in the comb at autopsy and consequently a

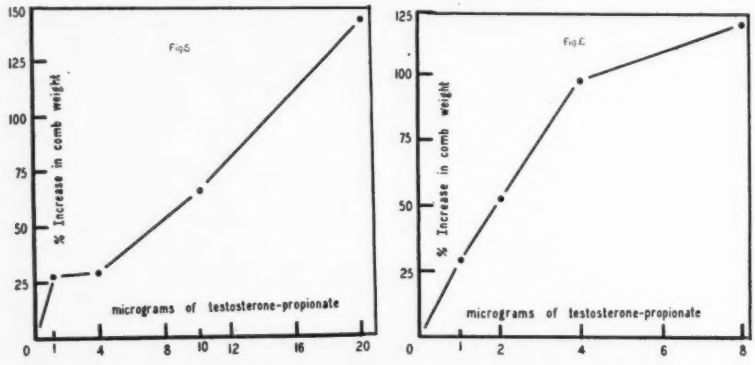


Fig.5
% Increase in comb weight
micrograms of testosterone-propionate
0 1 4 8 10 12 16 20
Fig.6
% Increase in comb weight
micrograms of testosterone-propionate
0 1 2 4 6 8

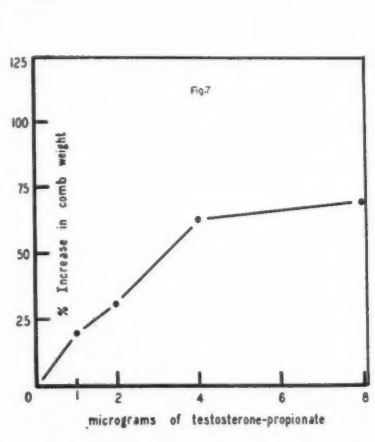
Fig. 5. Sixty-four chicks, 20-day-normal-diet birds caponized at 5 days of age and given comb injections of testosterone propionate on *days 10, 12, 14, 16, 18*. No priming injection. Fig. 6. Sixty-four chicks, 20-day-normal-diet birds caponized at 5 days of age and given 5 μg. of testosterone propionate as a priming dose on 8th day followed by the same androgen on *days 10, 12, 14, 16, 18*, injected into the comb.

further modification of technique was made by administering the male hormone only on alternate days. The curve for these results is shown as *B*.

Analysis of the figures in *B* show that comb injections on alternate days greatly improve the value of the test. A total of 2.5 μg. produces greater comb increment that does 5 μg. administered daily and a total of 5 μg. given on alternate days also stimulates more comb growth than the 10 μg. dosage shown in the *A* curve. The plateau in response, so evident in the daily injection, is not present although the response at the higher dosage is proportionately somewhat less than with the smaller quantities of hormone. The conclusion must be made that injections into the comb on alternate days is an efficient method for the determination of small amounts of androgen. It also appears probable that even with the small amounts of oil used that the quantities should be lessened still more and this point is being investigated still further by making injections on every third day instead of on alternate days.

The most striking results to be reported in this paper were those which appeared when the comb injections were made on alternate days in chicks which had been caponized on the 5th day after hatching. The curves for the baby capons are shown in figures 5 and 6 and in this instance, the hormone was administered on alternate days from the 10th to the 18th day and the animals were killed on the 20th. The experi-

mental results shown in figure 5 are different from the other series inasmuch as no priming dosage was given. The growth of the comb was remarkable, however, even in the absence of this priming, because there was significant comb growth at the 1 and 4 μg. levels of treatment. The curve for the 4, 10, and 20 μg. dosages is almost a straight line and it seems quite likely that there was an experimental error present in the 1 μg. series, perhaps in the preparation of the solution for injection. If a great deal of weight were to be placed upon these experiments a much more detailed analysis of the 1 and 4 μg. series would have to be made. Fortunately, however, this is unnecessary because the results which were observed after the injection of androgen into capons which did receive a priming dosage on the 8th day and subsequent comb injections on alternate days are much more critical. The data for these series are presented in figure 6.



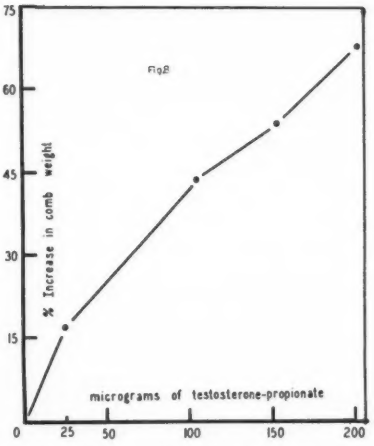


Fig. 7. EIGHTY BIRDS, 20-day-normal-diet females which received 5 μg. of testosterone propionate as a priming dose on 8th day with subsequent injection into the comb of the same androgen on *days* 10, 12, 14, 16, 18. Fig. 8. ONE HUNDRED AND 97 CHICKS were injected when received at 12 hours after hatching, given 4 additional injections at 12-hour intervals for a total of 5 injections and killed 20 hours after last treatment. All injections were made subcutaneously. Chicks were kept in boxes without food but were given 1 cc. of 0.9 normal saline solution at the same time the androgen was administered.

The comb increment ranges from 30.1 per cent following the administration of 1.0 μg. of the hormone to 120.2 per cent at the 8 μg. dosage. Comparison of these results with those for the 15-day-limited-diet experiments, figure 4, *B*, shows that the capon test will average about 100 per cent more effective. Actually the 1, 2 and 4 μg. dosages give almost a straight line response and all of the responses are significant. There is some evidence of a decreasing response at the 8 μg. level, but this may also be corrected with improvement in the method. A method for the assay of small amounts of androgen seems best satisfied by the use of the baby capon. The operation, although not easy, is no more difficult than the hypophysectomy of a rat. There are those who hesitate to abandon the use of a castrate animal in their assay work because there may be a possible influence of the animal's own gonads, if unoperated animals are employed. In this instance, we have a satisfactory form in which small amounts of male hormone can be detected. It should be emphasized, however, that it will probably be possible to improve upon the procedure as outlined here and this is being investigated further in our laboratory.

Two additional methods of assay were found to possess some merit and to be worthy of future consideration. Normal-diet pullets were injected in the same way as the capons with a priming dose on the 8th day and subsequent alternate-day-injections. The data which were given relative to comb growth in the pullets indicated an increase in relative comb size of only 1.7 times for the 10th to 20th day in the normal birds and it was logical to expect that any indirect effect through the inhibition of the pituitary by the injected androgen would have less effect than in males. Small amounts of testosterone propionate were administered to test the possibility of the female comb response and the results are shown in figure 7. There is a definite and significant response to 1 μg. of the male hormone which produced a 20.2 per cent increase and a definite plateau above the 4 μg. dosage level. An analysis of this plateau has been reserved for future consideration. Comparison of these data with those for the capon and the 15-day-limited-diet chicks indicates that the pullets are approximately about 50 per cent as effective as the capon in their responses to the 1, 2 and 4 μg. dosages, and hence would be satisfactory in many cases for androgen assay. The females are a little more responsive at the lower dosages, 4 μg. and under, than are the limited-diet chicks, but the plateau in their response makes them a doubtful value above 4 μg. although they are still slightly more responsive at 8 μg. than the limited-diet birds.

The excellent results in the study of gonadotropic hormones which have been obtained with the use of chicks kept in their shipping boxes without food or water appeared to present a method for possible assay of male hormone. The responses of chicks have been shown (10, 11) to have a very low standard error and although the differences between series is often not great, the data have proven to be highly significant on the basis of 'Student's' t test. The chief disadvantage in this procedure is the fact that about 90 hours is the maximum time that chicks can be expected to survive this treatment after which they die rapidly. That they do very well previous to this time is evidenced by the fact that 200 chicks were injected in the series shown in figure 8 and 197 of these survived in good condition at the termination of the experiment at 80 hours after the initial injection. It has been shown (unpublished data) that the comb may be composed of as much as 80 per cent water after treatment with androgens; for this reason injections of 1.0 cc. of water were given the chicks at the time the androgen was administered. The injections of both water and hormone were made subcutaneously but at different sites.

The data are satisfactory as a preliminary approach because the responses were good, even though relatively large doses of hormone were administered (25 μg. to 200 μg.). The range in response was from 18 per cent at the lowest level to 67 per cent at the highest. Some measure of the value of the averages may be gained by the fact that at 25 μg. the average of 18 per cent mean difference had a standard error of only ± 1.08 per cent. This test, therefore, may have some very practical application as a short and time saving method for the determination of small amounts of male hormone. The possibility of comb injections into the birds at this age is being investigated as well as some additional modifications in technique. The relatively small amount of variation strongly recommends the method for careful investigation.

The primary aim in the presentation of these data was to demonstrate that the chick can be used for the determination of small amounts of androgens. Several aspects of the problem have been omitted from the present discussion in order to present as clearly as possible that such assays are possible. No data were included on the nature of the comb growth although we have at present, and will publish, considerable material relative to the actual comb changes which are involved. Even more important are the statistical determinations. Determinations were made of the standard

errors and coefficients of variation and 'Student's' t test was employed to determine the validity of the data. No statements have been made which were not justified by these statistical methods. One problem which must be considered is the justification for use of the comb weight/body weight percentage and an evaluation of the data as suggested by Frank and his co-workers of the eviscerated animal. It may be stated briefly that the results have been more consistent when the comb as a percentage of body weight was used and there was no improvement in the relative values of the figures when evisceration was done. This may be due in part to the fact that we remove the feed from all animals 12 hours before autopsy and the viscera are remarkably constant in weight. Determination of the curves on the basis of response against the logarithm of dose has not been reported nor the regression curves determined. These as well as the above points have been left until further detailed analyses of one or two of the best methods reported in this paper are made using additional material.

SUMMARY

A study was made of 1318 White Leghorn chicks which were administered testosterone propionate or testosterone in dosages ranging from 1 to 1000 micrograms. Cockerels and pullets which were given limited amounts of food (limited diet) gave significant responses to subcutaneous injection but normal-diet cockerels had smaller combs than the controls at dosage levels of 25 to 50 micrograms. Administration of 100 micrograms, however, produced a significant growth. This variation was apparently the result of decreased androgen secretion by the animals' own testes which at low dosages more than offset the effect of the injected androgen. Limited-diet birds, in contrast, responded as though they were essentially castrates, therefore the problem of endogenous secretion was eliminated. Injections of androgen into the comb was efficacious, especially when administered on alternate days. A total of 2.5 micrograms produced a comb increase of more than 32 per cent. Chicks caponized at 5 days and given comb injections on alternate days had comb increments of more than 31 per cent at 20 days of age after administration of only one microgram of hormone. Normal-diet pullets similarly treated, although somewhat less responsive, also gave significant increases with as little as one microgram of testosterone propionate.

A short assay method was investigated in which the birds were injected at 12 hours after hatching and autopsied at 80 hours. These animals were kept without food or water and gave positive response to amounts of hormone ranging from 25 to 200 micrograms. The percentage increase, although not as great as in other series, had very low standard errors and the test is worthy of further detailed study.

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EFFECT OF CRYSTALLINE ESTRIN IMPLANTS ON THE PROXIMAL TIBIA AND COSTOCHONDRAL JUNCTION OF YOUNG FEMALE RATS

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INTRODUCTION

OSSIFICATION OF THE MARROW CAVITY of the long bones in pigeons that coincided with the maturation of the ovarian follicle was noted by Keyes and Potter (1) in 1934. Within a short time Zondek (2), using the domestic fowl and rat, and Gardner (3) working with the mouse, reported essentially similar results following the administration of estrogens. Since then a number of workers have confirmed and elaborated the rôle of the estrogens in calcium metabolism and their effects on bone morphology.

Considering the number of different species investigated, the pigeon (1, 4, 5), the common fowl (2, 6), the duck (5), the sparrow (7), the rat (2, 8, 9, 10), the guinea pig (11), and the mouse (3, 12, 13), a surprising degree of similarity in results has been observed. In general it has been found that administration of excess amounts of estrogens over a relatively long period of time causes a hyper-ossification of the long bones; and where young growing animals were the subject of investigation, a stunting of growth was observed.

The differences that have been noted are differences in the manner of ossification, i.e., endochondral, endosteal, or a combination of both. Gardner and Pfeiffer (3) and Sutro (12) reported a marked stimulation of endosteal bone formation in the mouse after prolonged estrinization. That the same is true for birds was shown by Zondek (2) Pfeiffer, Kirschbaum, and Gardner (7), and Bloom, Bloom, Domm and McLean (5), working with the fowl, sparrow and pigeon, respectively.

In the rat, endosteal bone formation after estrinization does not occur (10). However, the histological picture of hyperossification does appear, since an increase in the number and length of the trabeculae extending beneath the proximal epiphyseal cartilage of the tibia was observed. Day and Follis (10) interpreted this excessive bone formation as the result of a retardation of the resorption that normally occurs as these trabeculae extend into the marrow.

Silberberg and Silberberg (13), however, report a final state of increased resorption that follows the initial stage of decreased resorption after prolonged estrinization in mice.

In addition to changes in bone formation noted after prolonged estrogen administration, changes in the histology of the epiphyseal cartilage have been noted. Gaarenstroom and Levie (8) have reported a shrinkage of the epiphyseal cartilages

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of the young rat after 14 days of estrinization. Day and Follis (10) found no change in the width of the proximal epiphyseal cartilage of the rat tibia after 17 days of treatment with estradiol benzoate. After 26 days, however, a narrowing of the cartilage plates of the estrogen-treated animals that continued over the 115 days of observation was noted. Sutro (12) noted a rapid narrowing of the epiphyseal plates in estrinized mice. Silberberg and Silberberg (13) observed a similar inhibition of epiphyseal cartilage growth in young mice after chronic estrogen treatment and also found that such treatment retarded epiphyseo-diaphyseal union. Zondek (2) likewise reported a retardation of epiphyseal "closure" in the rat after prolonged estrogen administration.

Except for the observations of Zondek (2), little correlation of the effects of estrogen on skeletal structures with more generally known aspects of estrin physiology has been made. Therefore, when a large series of animals at this laboratory was subjected to high levels of estrogen to determine the effects on the hormone production (rate of secretion and content) of the pituitary, an investigation of the bone histology of these animals was included. The state of the endocrine organs of the estrinized rats and the response of the endocrine system of immature hypophysectomized rats and of immature pigeons implanted with the pituitaries of the estrinized rats will soon be reported.

MATERIALS AND METHODS

Six rats were selected from each of 5 groups of animals that had received implants of estrin tablets, 3 experimental and 2 control (table 1). The first group received 1 pellet of alpha-estradiol dipropionate (10 mg.) subcutaneously on the 71st day of age and was autopsied 7 days later. The second group received 3 weekly implants of the same type of estrogenic pellets beginning on the 54th day and were autopsied on the 75th day. The third was a control group for the preceding two, 6 normal rats autopsied on the 76th day. The remaining 12 animals were made up of an experimental group of 6 rats that had received 6 weekly implants beginning on the 54th day and a control group of 6 normal animals autopsied at the same age, 96 days.

The carcasses were fixed in 10 per cent neutral formalin. The right tibia and the fourth right rib were dissected and prepared for histological examination (14). Care was taken to cut sagittal sections serially of both the tibia and the costochondral junction. Central sections were carefully chosen and formed the basis of the study reported.

RESULTS

Histological Findings

Tibia. The histology of the epiphyseal junction of a normal rat of 76 days is well shown in figure 1, Plate I. The cartilage plate displays the remnants of embryonic resting hyalin cartilage on its epiphyseal side from which orderly columns of proliferating cartilage cells develop. These columns of cells record the progressive stages of proliferation, vesiculation, and capillary erosion that each cell eventually reaches. Above the epiphyseal disc, heavy, bony plates of the epiphysis are visible, while below numerous trabeculae of calcified cartilage project toward the diaphysis to serve as the framework of the primary spongiosa. The number of trabeculae in a horizontal plane diminishes as the center of the diaphysis is approached. At the zone of erosion, a thick layer of osteoblasts surrounds the invading capillaries and lies in apposition to the newly formed trabeculae of the primary spongiosa.

One subcutaneous implant of a 10 mg. estrin pellet produced in 7 days the effects seen in figure 2, Plate I. A marked shrinkage of the epiphyseal cartilage is apparent.

TABLE 1

Group	Animal Number	Age at Autopsy	Weight at Autopsy	Width of Epiphyseal Cartilage of Tibia	Width of Costo-Chondral Growth Disc
		days	*gm.*	*micra*	*micra*
Normal control	GH4804	75	192	300	304
	G4827	75	186	270	278
	W8200	76	184	255	322
	B8211	76	200	270	330
	W8257	76	204	230	278
	W8176	76	170	220	287
	m=		**189**	**257**	**300**
1 Implant, on 71st day of 10 mg. estradiol dipropionate subc.	BH8001	78	158	105	191
	B8014	78	150	135	174
	W8129	78	168	105	157
	BH8145	78	181	155	157
	G7995	78	141	140	218
	G8099	78	116	120	174
	m=		**152**	**125**	**178**
3 Weekly implants, from 54th day, of 10 mg. estradiol dipropionate subcutaneously	B4947	75	176	190	235
	W5048	75	194	140	174
	BH5059	75	174	180	218
	GH6061	75	149	155	200
	W4875	75	140	220	218
	GH4990	75	164	125	157
	m=		**166**	**168**	**191**
Normal control	W4300	96	184	155	130
	B3202	96	256	180	174
	B5728	96	230	185	174
	BH5743	96	216	180	183
	G4259	96	222	180	174
	W4292	96	244	180	191
	m=		**225**	**176**	**176**
6 Weekly implants, from 54th day, of 10 mg. estradiol dipropionate subcut.	G3400	96	192	105	148
	BH3527	96	164	100	113
	B3431	96	202	105	104
	B3537	96	201	120	104
	W3489	96	218	155	130
	B3494	96	186	140	122
	m=		**194**	**120**	**121**

Measurements reveal a narrowing of more than 100 micra (table 1). Osteoblastic activity is somewhat diminished when compared with the normal but considerable activity is still visible.

In many respects, the cartilage histology resembles that of an animal that had been almost starved during the experimental period. That this is more than likely is shown by the loss in weight suffered by this group (table 1). Although the food intake of these animals was not measured, gross observations revealed a marked anorexia.

The group that received 3 weekly implants of 10 mg. pellets of estrin (fig. 3, Plate I) reveals a striking picture of hyperossification. The epiphyseal disc has an essentially normal histology that denotes a recovery in activity. During the 2nd and 3rd week of the experimental period, measurement of the food consumption of this

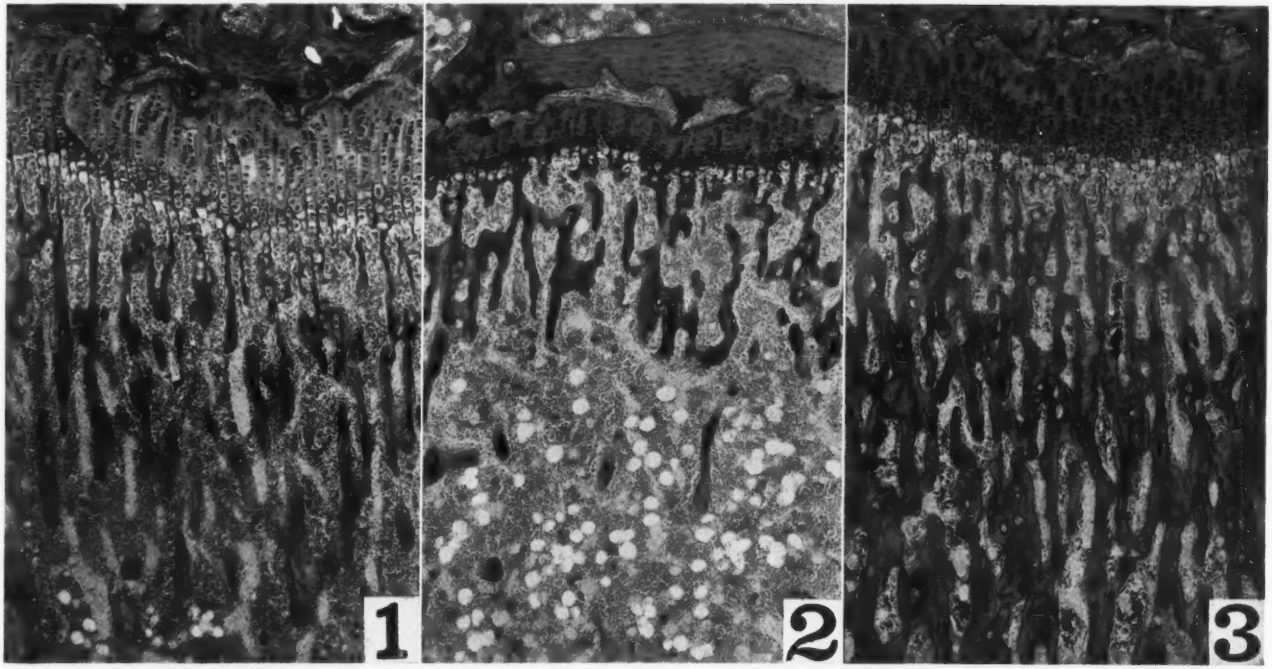

Plate I. Proximal epiphyseal cartilage of right tibia of rat. Central sagittal section. Hematoxylin and eosin. 10 micra. ×66.

Fig. 1. Normal; 76 days of age at autopsy.
Fig. 2. One implant, 71st day, 10 mg. estradiol dipropionate subcutaneously; 78 days of age at autopsy.
Fig. 3. Three weekly implants from 54th day, each, 10 mg. estradiol dipropionate subcutaneously; 75 days of age at autopsy.

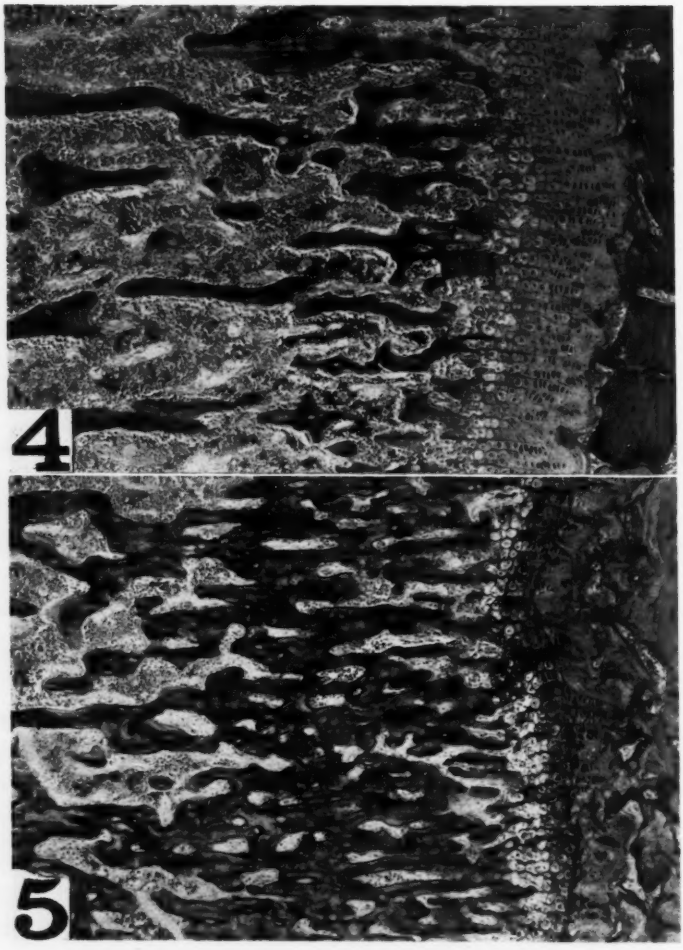

PLATE II. PROXIMAL EPIPHYSEAL CARTILAGE OF RIGHT TIBIA OF RAT. Central sagittal section. Hematoxylin and eosin. 10 micra. ×70.

Fig. 4. Normal; 96 days of age at autopsy.

Fig. 5. Six weekly implants from 54th day; each 10 mg. estradiol dipropionate subcutaneously; 96 days of age at autopsy.

group revealed no significant difference between that of the experimental and that of the control. The shrinkage that is revealed by micrometer measurements (table 1), therefore, may be the result of the estrogen treatment alone or the residual effect of the initial reduction in food consumption. Extending far below the cartilage is a mass of thick anastomosing trabeculae that obliterates the upper fifth of the marrow space that normally occupies the shaft of the bone. Marked osteoblastic activity is apparent. No significant effect on periosteal or endosteal activity was detectable.

The bone histology of the 96-day normal control (fig. 4, Plate II) is essentially the same as in the normal control of 76 days. The diminution in cartilage width and ac-

Plate III. Costochondral junction of 4th right rib of rat. Central sagittal section. Hematoxylin and eosin. 10 micra. ×70.
Fig. 6. See legend figure 1. Fig. 7. See legend figure 2. Fig. 8. See legend figure 3.

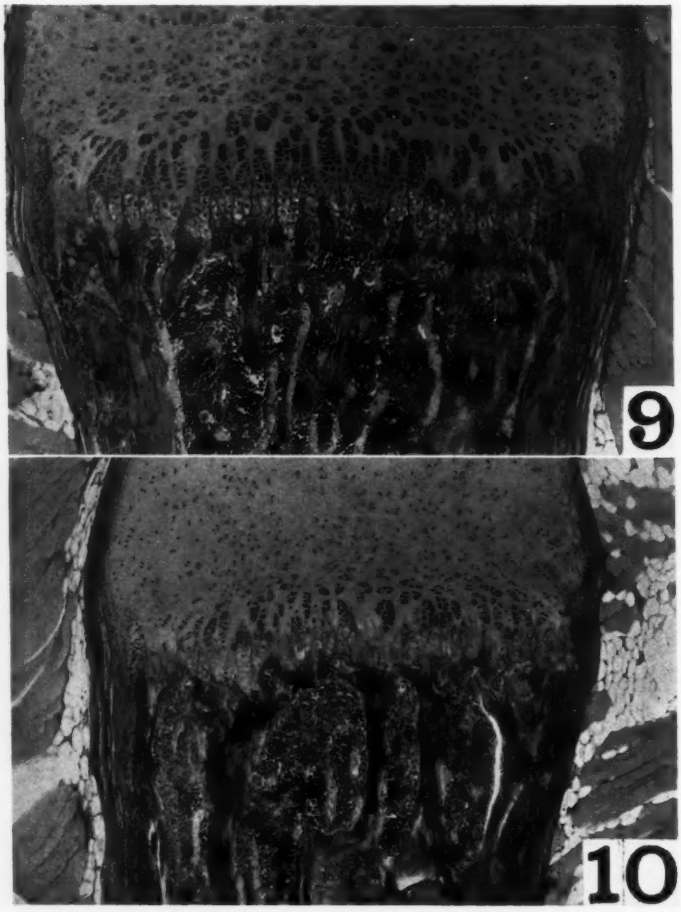

PLATE IV. COSTOCHONDRAL JUNCTION OF 4TH RIGHT RIB OF RAT. Central sagittal section. Hematoxylin and eosin. 10 micra. ×73.

Fig. 9. See legend figure 4. Fig. 10. See legend figure 5.

tivity at the erosion zone are signs of increasing maturity. The experimental group that received 6 weekly implants of estrin pellets (10 mg. each) reveals effects that are quite similar to those seen in the group receiving 3 weekly implants. In this case, the shrinkage in cartilage width of over 50 micra (table 1) is more likely due to the excess estrogen or an endocrine imbalance caused by it than to a reduction in food consumption. However, the absence of complete data on food consumption makes necessary the consideration of a possible reduction in food intake. As for the distribution of osseous tissue the trabeculae nearer the cartilage disc show a greater degree of coalescence while those further away show some signs of resorption and reorganization into thicker and more widely spaced bony plates. Definite, clear cut signs of resorption of the lower trabeculae were not consistently shown in all of the animals

of this group. While the trabeculae projecting most deeply into the marrow cavity are frequently more widely separated than those nearer the epiphyseal cartilage (fig. 5) deeper penetration of the diaphyseal marrow cavity was present in the 6-week group than in the 3-week group. Therefore, the extent of hyper-ossification was correlated with the length of estrin treatment.

The ribs. The costochondral junction (fig. 6, Plate III) is similar in many ways to the epiphyseo-diaphyseal junction of the tibia. The cartilage in juxtaposition to the osseous portion of the rib has the same pattern of orderly rows of cells undergoing the changes associated with endochondral bone formation. Plates III and IV of the costochondral junction encompassing as they do the entire region involved, offer a more complete picture of the effects of estrinization. The total width of the bone and the periosteum is well shown.

The group receiving one weekly implant of estrin (fig. 7, Plate III) reveals first a loss in the total dimension of the rib at the costochondral junction. The region of cartilage cells undergoing changes preceding bone formation, therefore, the growth disc, is hypoplastic (table 1). The terminal cells of the cartilage columns do not display the degree of vesiculation seen in the normal control of that age. The cartilage columns seem to have been invaded more deeply by capillaries than in the normal controls. Osteoblastic activity has not been affected as shown by the trabeculae beneath the cartilage and by the extent and thickness of the periosteum on either side of the growth disc.

The group receiving 3 weekly implants of estrin pellets shows a greater loss in the total dimensions of the rib (fig. 8, Plate III). This response is correlated with the administration of estrin at an earlier date and the stunting of growth that began at that time. The growth disc of the costochondral junction of this group does not differ markedly from that of the one-week group (table 1). Again the extensive formation of bony trabeculae and the appearance of the periosteum on either side of the growth disc reveal no diminution of osteoblastic activity.

The group receiving 6 weekly implants of estrin and its control are pictured in figures 9 and 10, Plate IV. In this case, the difference in size is most striking. In the experimental group the regressive changes of the cartilage growth disc are the most marked of all the groups examined (table 1). The irregularly arranged bony plates that lie in juxtaposition to the growth disc reveal an abnormally deep penetration of the columns of cartilage cells. The sealing off of the growth disc by bone in this group brings to mind the effects of hypophysectomy. However, the irregular pattern of these bony plates and the presence of excessively heavy, long trabeculae extending beneath the growth disc rule out any true similarity between the effects of estrinization and hypophysectomy. The retention of considerable osteoblastic activity is another dissimilar point.

SUMMARY

High levels of estrin, in one week, caused an involution of the proximal growth cartilage of the tibia of young adult female rats. After three weeks' treatment the epiphyseal cartilage partially recovered its growth capacity and a striking mass of thick trabeculae filled the upper fifth of the diaphyseal marrow cavity. After 6 weeks of estrinization, some signs of resorption and reorganization into thicker, more widely spaced trabeculae were noticeable at the distal end of the mass of trabeculae. Increased periosteal or endosteal activity was not observed in any estrinized rat.

After estrinization, the growth disc of the costochondral junction displayed a series of regressive changes that increased in severity with the prolongation of treatment. The initial regressive change followed by a recovery in growth activity as

seen in the epiphyseal disc of the tibia was not as marked in the costochondral junction. Excessive formation of bony trabeculae that obliterate the marrow cavity was not apparent in the costochondral junction although thicker and longer trabeculae were found beneath the growth disc of treated rats than are found in the controls. Irregular bony plates in juxtaposition to the growth disc that seal off the cartilage cell columns were seen in the costochondral junction and not in the epiphyseal cartilage of the tibia after 6 weekly implants of estrin pellets.

The principal distinguishing characteristic of the reaction in the tibia and rib was the difference in amount and distribution of osseous tissue after estrogen treatment.

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THE PHYSIOLOGICAL SIGNIFICANCE OF THE ESTROGEN:PROGESTERONE RATIO ON VAGINAL CORNIFICATION IN THE RAT

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THERE HAS BEEN CONSIDERABLE CONTROVERSY over the apparent 'inhibitory' or 'antagonistic' actions between the two ovarian hormones, estrin and progesterone. The effects of these hormones has been said to depend upon the quantitative ratio between them. When this ratio of estrin to progesterone was optimal the physiological effect was synergistic; however, when it was altered in one direction or another the effect of one hormone was considered to be 'inhibited' and the other to prevail.

Parkes and Bellerby (1) found that 2 to 5 times as much estrogen was required to produce estrous changes in the vagina of a lactating mouse as in the castrated mouse, and Loeb and Kountz (2) showed that in the guinea pig vaginal cornification could not be produced by estrogen during pregnancy. Courrier (3) was unable to produce a typical estrous smear in a postpartum guinea pig by the administration of 190 R. U. of "estrogen," and Selye and McKeown, in 1935, failed to produce vaginal cornification in lactating rats with the daily injection of 3000 micrograms of estrone (4).

Allen and Meyer carried out a series of experiments on castrated mice. They concluded that more RB. U. of progesterone than R. U. of estrogen were necessary in order to suppress completely the cornification of the vagina and the estrous smear (5). Korenchevsky and Hall (6) using doses of estrin and progresterone over a period of 20 to 21 days varying from 1 microgram of estrone 3 times a week to 30 micrograms daily and 0.5 to 3.0 mg. of progesterone, concluded that in the ovariectomized rat excessive doses of estrogen were antagonistic to the cooperative action between the 2 ovarian hormones and that the most physiological ratio was 1 μg. of estrone daily to 1.5 mg. of progesterone. Courrier and Gross (7) found it necessary to give 8 mg. of progesterone in a single injection to suppress the estrous smear obtained with 0.02 mg. of estradiol. From this observation they state that the physiological dose of progesterone must be from 200 to 400 times that of the estrogen. Selye, Browne, and Collip, in 1936, were able to produce diestrous smears in 5 of 6 castrated rates by the daily injection of 0.4 mg. of progesterone in spite of the simultaneous injection of 30 μg. of estrone (8).

It can readily be seen from the data of Selye and McKeown on one hand (4) and Courrier and Gross on the other (7), that were these ratios correct it would be necessary for the corpus luteum of lactation in the rat to produce 3 gm. of progesterone a day. As such a figure seemed fantastic it was thought that an explanation might be found if the progesterone was administered in a more physiological manner. The present experiments were undertaken accordingly.

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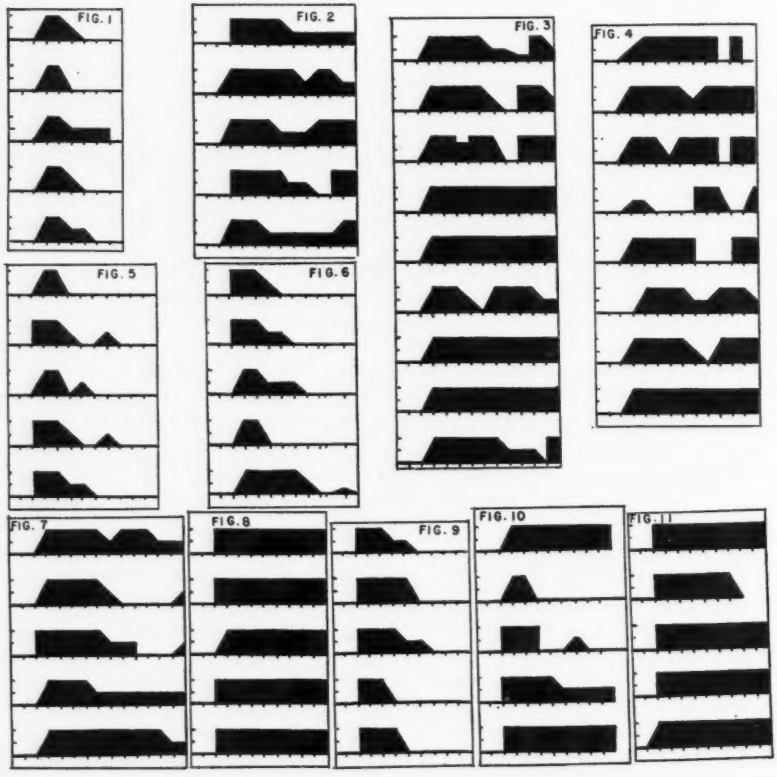

Fig. 1. ONE BLOCK ON THE ABSCISSA represents 1 day and 2 blocks on the ordinate represent a full estrous smear. The rats received simultaneous doses of 0.1 μg. of estradiol and 1 mg. of progesterone daily. Four of the 5 rats were in diestrus by the 6th day after the beginning of estrus. Estrogen:Progesterone = 1/10,000. Fig. 2. THESE RATS RECEIVED SIMULTANEOUS DOSES of 0.1 μg. of estradiol and 0.1 mg. of progesterone. This chart does not show any essential variation from figure 4 in which the rats received no progesterone. It is therefore assumed that the variation found in the vaginal smears is not due to progesterone but that 0.1 μg. of estradiol is too small a dose of estrogen to maintain a continuous vaginal estrus. Estrogen:Progesterone = 1/1000. Fig. 3. THESE RATS RECEIVED 0.1 μg. of estradiol and 0.02 mg. of progesterone. Here again there is no visible progesterone effect and the chart resembles figure 4. Estrogen:Progesterone = 1/200. Fig. 4. THESE RATS RECEIVED NO PROGESTERONE and only 0.1 μg. of estradiol daily. In only 1 animal was vaginal estrus maintained continuously. This is apparently too small a dose of estrogen to maintain an even estrous smear.

Fig. 5 and 6. THESE ANIMALS RECEIVED 100 TIMES the dose of estradiol and the same amount of progesterone as the animals in figure 1, e.g., 10μg of estradiol and 1 mg. of progesterone. The results, however, are identical, demonstrating that the estrogen:progesterone ratio has no bearing on the vaginal response when both hormones are administered simultaneously over a period of days. Estrogen:Progesterone = 1/100.

Fig. 7. THESE ANIMALS RECEIVED 10 μg. of estradiol and 0.5 mg. of progesterone daily. Only 2 of these rats showed diestrous smears during the injection period. 0.5 mg. of progesterone therefore appears to be an inadequate dose of progesterone to produce vaginal diestrus in the majority of rats. Estrogen:Progesterone = 1/50. Fig. 8. THESE RATS RECEIVED DAILY DOSES OF 10 μg. of estradiol and 0.1 mg. of progesterone. The progesterone dosage in this case was completely inadequate. Estrogen:Progesterone = 1/10.

Fig. 9. THESE RATS RECEIVED 100 μg. of estradiol and 1 mg. of progesterone daily. The estrogen:progesterone ratio was therefore 1:10 and in this instance all of the animals came into vaginal diestrus promptly, although in figure 10 a similar ratio was seen to be entirely inadequate. It is therefore demonstrated that the dose level of progesterone is the important factor rather than the estrogen:progesterone

EXPERIMENTAL PROCEDURE

Four groups of oöphorectomized rats were injected daily with progesterone[1] in doses of 0.02 mg., 0.1 mg., 0.5 mg., and 1 mg. respectively. In each group estradiol was administered simultaneously in amounts of 0.1 μg. to 100 μg. Daily vaginal smears were taken and at the end of the injection period the rats were killed and histological studies made on the vaginal mucosa. The details of the experiment are given in table 1 and charts of the individual rat smears can be seen in figures 1 through 11. The histological appearances of the vagina are shown in figures 12, 13, and 14.

Those rats in *group 4* which received 1 mg. of progesterone, regardless of the amount of estradiol given simultaneously, showed diestrous or modified smears. The majority of animals receiving only 0.5 mg. of progesterone also exhibited diestrous or modified

TABLE 1. TABULATION OF EXPERIMENTAL RESULTS

Group	Progesterone	Estradiol	Duration of Injection	Number of Animals	Type of Vaginal Smear		
					Cornification continuous	Modified	Diestrous
	mg.	*μg.*	*days*				
Control	0	0.1	12	8	2	6	0
1	0.02	0.1	12	9	4	5	0
2	0.10	0.1	12	5	1	4	0
	0.10	10.0	10	5	5	0	0
	0.10	100.0	10	5	5	0	0
3	0.5	10.0	12	5	0	3	2
	0.5	100.0	10	5	2	1	2
4	1.0	0.10	8	5	0	1	4
	1.0	1.0	25	5	0	5	0
	1.0[1]	1.0	12	5	0	5	0
	1.0	10.0	12	5	0	0	5
	1.0[1]	10.0	12	5	0	0	5
	1.0	100.0	10	5	0	0	5

[1] Progesterone given in 2 doses daily.

smears. No rats injected with less than 0.5 mg. of progesterone had diestrous smears or vaginal mucification. Those animals which received only 0.1 μg. of estradiol showed a tendency to have modified smears suggesting estrous cycles. As this reaction was also noted in a control group of rats receiving 0.1 μg. of estradiol and no progesterone, it was thought that these animals were being maintained on the minimal amount of estradiol which is capable of producing vaginal cornification. The inherent vaginal cycles described by del Castillo and Calatroni (9) and by Zuckerman (10) were, therefore, probably being manifested and the changes should not be attributed to the action of the progesterone.

[1] The authors wish to express their appreciation to the Ciba Corporation, Summit, New Jersey, for kindly supplying the progesterone used in these experiments.

ratio. Fig. 10. THESE RATS RECEIVED 100 μg. of estradiol and 0.5 mg. of progesterone daily and again 2 out of 5 animals showed diestrous smears during the course of injections. In this case the estrogen: progesterone ratio was 1:5.

Fig. 11. THESE ANIMALS RECEIVED 100 μg. of estradiol and 0.1 mg. of progesterone daily. One animal showed leucocytes in the smear on the 8th day of injection; histologic studies showed no mucification. All other rats remained in constant vaginal estrus.

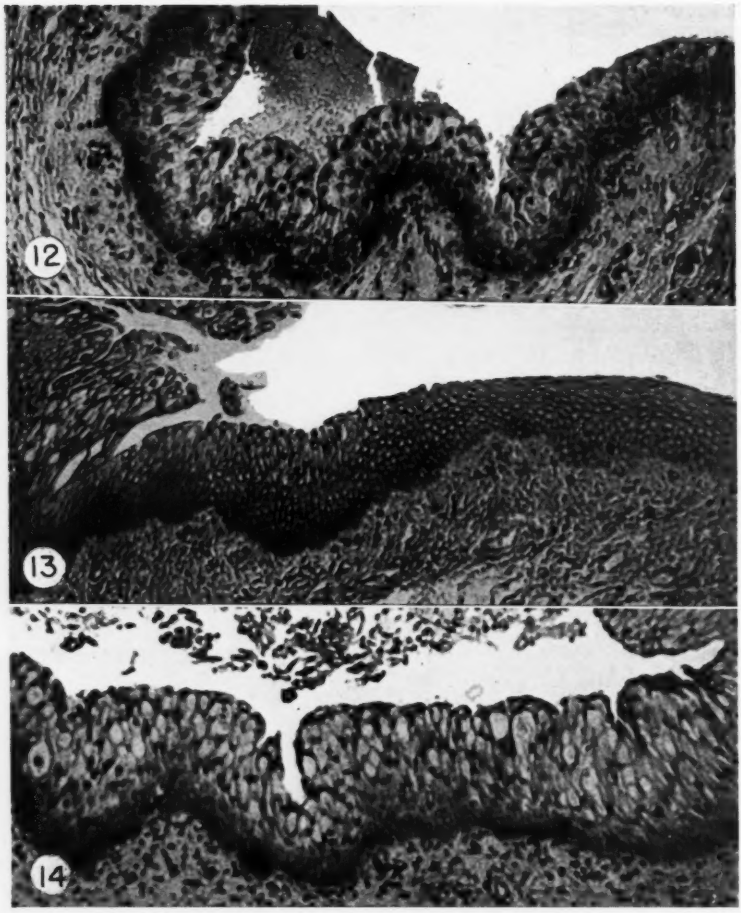

Fig. 12. PHOTOMICROGRAPH OF A SECTION OBTAINED FROM THE VAGINA of an oöphorectomized rat which had received 1 μg. of estradiol and 1 mg. of progesterone daily for a period of 25 days. The animal came into diestrus on the 6th day after the appearance of the estrous smear and had nucleated cells in the smear on 7 days thereafter. The smear had been pure diestrus for 8 days. There is mucification of the top layer of epithelial cells.

Fig. 13. PHOTOMICROGRAPH OF A SECTION OBTAINED FROM THE VAGINA of an oöphorectomized rat which had received simultaneous injections of 10 μg. of estradiol and 1 mg. of progesterone daily over a period of 10 days. The rat had been in diestrus 4 days. The mucification in this case is much more marked than in figure 12.

Fig. 14. PHOTOMICROGRAPH OF A SECTION TAKEN FROM THE VAGINA of an oöphorectomized rat which had received simultaneous daily injections of 100 μg. of estradiol and 1 mg. of progesterone over a period of 11 days. This animal came into diestrus 4 days after the appearance of the estrous smear. The vaginal mucification in this case is greater than in either of the other 2 cases although the amount of estrogen has been increased 100 times. In this circumstance at least there is no 'antagonism' between large amounts of estrogen and progesterone.

A single group of 5 rats was injected with 1 mg. of progesterone daily for 4 days prior to the estradiol administration. Ten μg. of estradiol was then given simultaneously with the progesterone over a 10-day period. This preliminary course of corpus luteum hormone did not prevent the appearance of the initial estrous smear and the animals as a whole showed no differences in reaction from the group of rats receiving 1 mg. of progesterone and 10 μg. of estradiol without the preliminary progesterone.

DISCUSSION

It would seem from these observations that there is a certain adequate dose of progesterone which when given with estrogen will convert an estrous into a diestrous smear and produce mucification of the vagina in the oöphorectomized rat, even in the presence of very large amounts of estrogen. Mucification, in fact, is much more highly developed in those animals receiving higher doses of estrogen (fig. 12, 13, 14). It is also apparent that, although a large amount of progesterone may be necessary to prevent the initial appearance of estrus, a much smaller amount of progesterone is capable of converting the estrous smear to the diestrous one if injections are continued over a period of days. Courrier's ratio of 1:200 or 1:400 would necessarily be changed under the circumstances of the present investigation to a ratio of 1:5 or 1:10. However, if higher doses of estrogen had been given this ratio might have been lowered even further. Of more importance than the quantitative ratio between the 2 hormones, therefore, is the absolute amount of progesterone. As 0.5 to 1.0 mg. of progesterone is adequate for mucification in the presence of any practicable dose of estrogen, this amount may correspond approximately to the corpus luteum hormone output of the rat ovary.

Hisaw, Greep, and Fevold (11) have shown that in the Macacus rhesus these 2 hormones are 'synergistic' or 'antagonistic' depending upon the end-organ concerned. Thus progesterone increased the effect of estrogen in enlarging the uterus but estrogen was apparently inhibited in its typical effects upon the cervix, vagina, and sex skin.

Gillman and Stein (12) have demonstrated that in the baboon the estrogen: progesterone ratio is not a fixed quantitative one but rather that the physiologic effect "depends upon an optimal concentration of progesterone." A physiological level in the baboon has been found to be as low as 2 mg. of progesterone when given with 10 mg. of estradiol benzoate.

The preliminary administration of adequate amounts of progesterone before estrogen administration has been found to have no action in hastening or modifying the subsequent changes which take place in the castrate rat vagina. This is further evidence that progestational changes are normally brought about by the 2 hormones acting together. It is therefore apparent that antagonism and inhibition are words which have no meaning in regard to the estrogen: progesterone relationship. The conversion of the estrous into the diestrous smear is not an inhibition of the estrogen effect or due to antagonism between the 2 hormones, but rather the production by the 2 hormones of an entirely different type of histological change. The diestrous smear occurs because the cornified vagina has been mucified, but without the original cornification true mucification would not have occurred at the progesterone dose level which was used in the present experiment.

SUMMARY

Estrogen and progesterone have been injected simultaneously into oöphorectomized rats over varying periods of time and the vaginal smears have been followed. The amounts of progesterone used were not sufficient to suppress the initial estrous smear. However, when the progesterone dosage was adequate the estrous smear was

immediately converted into the diestrous type and remained so throughout the injection period regardless of the amount of estrogen given. It therefore seems that the ratio of the 2 hormones bears no relationship to the appearance of the diestrous smear. If the dosage of progesterone is adequate, the cornified smear can be converted to the diestrous type even in the presence of large amounts of estrogen. The administration of progesterone over a period of time before beginning the estrogen does not prevent the initial cornification.

As far as can be determined, this reaction constitutes the only practicable biologic test by which progesterone may be detected in the presence of large amounts of estrogen. Unfortunately, however, it is not specific for corpus luteum hormone as androgens are equally effective in modifying the vaginal effect of estrogen. For an assay method therefore, the rat vagina suffers in this regard the same disadvantage as the rabbit endometrium.

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THE INTERACTION OF THE OVARIAN HORMONES IN EXPERIMENTAL MENSTRUATION[1,2]

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ALTHOUGH THE SPECIFIC CAUSE responsible for the precipitating uterine bleeding during the normal menstrual cycle is yet quite obscure, it seems probable that an explanation will be found in the physiological action of estrogen and progesterone on the endometrium. From an experimental standpoint, there is general agreement that bleeding will occur following an adequate estrogen treatment if the injections are discontinued or the dosage reduced below a certain threshold level. Such bleeding can be inhibited indefinitely by giving progesterone and is precipitated by its withdrawal. It is also a general experience that once an endometrium has been transformed into a fully developed progestational condition by progesterone it is extremely difficult if not impossible to prevent bleeding by giving estrogen. The exact progestational effects resulting from a series of injections of progesterone, which make it difficult for estrogen to inhibit bleeding, are not known but they are not correlated with modification of the uterine glands. The simultaneous injection of progesterone for a surprisingly short time during an estrogen treatment may, following its withdrawal, precipitate bleeding in the presence of estrogen. The purpose of this paper is to present the results of a more detailed study of the effects of progesterone on the ability of estrogen to maintain the endometrium.[3]

PROCEDURE

Castrated adolescent monkeys (*Macaca mulatta*) were given continuous treatments with 1000 I.U. of estradiol daily. Between the 20th and 30th day 1 I.U. of progesterone was injected daily for periods of 1 to 5 days while the estrogen treatment was continued uninterruptedly. The estradiol was dissolved in sesame oil and injected subcutaneously in a single daily dose while progesterone, also dissolved in sesame oil, was divided in some instances into two equal doses and given twice daily, and in others it was given in a single daily dose with entirely comparable effects. The injections of estradiol were usually stopped for a few days during the period of bleeding which followed the discontinuance of progesterone, after which the animals were again given estradiol for at least 20 days before being subjected to another progesterone treatment. Changes in the vaginal smear and the degree of edema and color of the sexual skin were recorded daily and the appearance and duration of bleeding determined by presence of macroscopic amounts of blood in the vaginal lavage.

EXPERIMENTAL RESULTS

Uterine bleeding. Several investigators have reported uterine bleeding in both castrated monkeys and women during continuous estrogen injections. Zuckerman (1)

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[2] We wish to thank the Schering Corporation, Bloomfield, New Jersey, for generous gifts of estradiol and progesterone used in these experiments.

[3] Consult, *Sex and Internal Secretions*, Williams and Wilkins, 2d Edition, Chapter VIII, for a recent review of pertinent literature.

found that bleeding may occur in castrated rhesus monkeys during continuous treatment with estrogen when the daily dose is below a certain threshold. This threshold, which varies in different animals, shows a cyclic fluctuation between about 50 and 250 I.U. Therefore, if bleeding is to be avoided during an estrogen treatment, the dosage must be maintained above the highest limit of threshold fluctuation. Zuckerman states that "about 200 I.U. of estrone a day is sufficiently above the threshold range in most animals for uterine bleeding not to occur during a period of 40 days of injections." We have mentioned that the monkeys in the experiments reported here were given about 1000 I.U. of estradiol (8.33μg.) daily. That 1000 I.U. daily is sufficiently above the threshold required to maintain the endometrium is shown by the fact that in a large number of experiments bleeding did not occur even when the treatment was continued for over 100 days.

Eight castrated animals were given 1 mg. of progesterone on 5 successive days between the 20th and 30th day of a chronic estrogen treatment. Four of these began bleeding on the 3d and 4 on the 4th day after the injections of progesterone were stopped. Four animals received similar treatment with the exception that the progesterone was injected for only 3 days. Three of these started bleeding on the 4th and one on the 5th day following the last injection of progesterone. In another group of 6 animals progesterone was injected on 2 days, and bleeding started on the following 5th day in 3 instances and on the 6th in two. The 6th animal had not bled at the end of 11 days following the progesterone injections, and a second 2-day treatment was given. This failed to cause bleeding within the next 23 days, whereupon 2 mg. of progesterone was given on a single day, and bleeding started 6 days later. In 7 animals, given only 1 mg. of progesterone on a single day, 4 began bleeding on the 5th, 6th, 7th, and 10th day respectively, while 3 had not bled by the 15th, 17th and 31st days.

Six animals, 5 of which had received 1000 I.U. of estrogen daily for 93 days and 1 for 82 days, were given 1 mg. of progesterone at a single injection and, although the estrogen treatment was continued, 3 began bleeding on the 9th, 1 on the 8th, and 1 on the 7th day thereafter. The 6th monkey (estrogen 93 days) had not bled by the 17th day, at which time she was given 1 mg. of progesterone on each of 2 successive days, and bleeding did not take place within the next 11 days. This was followed by a single injection of 2 mg. of progesterone without effect by the 7th day, whereupon 1 mg. was given on 3 consecutive days, and bleeding started 5 days later.

In another series of four animals, 2 intradermal injections of 0.25 mg. of progesterone in 0.05 cc. of oil were given simultaneously in separate areas of the sexual skin on the 29th, 30th, 30th and 49th days respectively during continuous estrogen treatment. Two of these began bleeding on the 4th and 7th days after the progesterone was administered, while the other 2 had not bled by the end of 28 days thereafter.

It is also known that bleeding will occur during an estrogen treatment if the dosage is suddenly reduced below a certain threshold amount (1). In view of this and other facts it seemed advisable to test the sensitivity of the endometrium to a withdrawal of estrogen under the conditions of these experiments. The animals employed for this purpose had been used repeatedly in the experiments described above. They had been on estrogen continuously for several weeks with the exception of brief rest periods during active bleeding. In four animals, the injection of estrogen (1000 I.U. daily) was omitted for 2 consecutive days following a 20-day treatment dated from the conclusion of the last experimental menstruation. Bleeding began in 1 animal before the end of the 48-hour-skipped period, and in 2, 3, and 4 days later, while in the 4th experiment no bleeding had occurred by the end of the 19 days, at which time treatment was again omitted for 2 days, and bleeding started 3 days after the estrogen

injections were resumed. In a 5th experiment, 2 days were skipped following a 50-day estrogen treatment, and bleeding was observed the morning of the 2nd day after the resumption of the injections.

The effects of discontinuing an estrogen treatment for 2 days were also tested in 4 experiments which were devised originally for another purpose. Two intact adolescent monkeys were given 3000 I.U. of estradiol daily for 21 and 22 days, respectively, after which they were unilaterally castrated and continued on 3000 I.U. of estradiol plus 5 R.U. of pituitary FSH daily for 10 days, at which time the remaining ovary was removed. This treatment was followed immediately by 3000 I.U. of estradiol daily for 5 days, at which time the dosage was reduced to 1000 I.U. daily for 12 and 13 days, respectively, after which 2 days were skipped, and both animals began bleeding on the 2nd day following the skipped period. The fact that the gonadotropic hormones used in these experiments induced follicular development without luteinization indicates that these animals were entirely under the influence of secreted and injected estrogen (49 days) previous to the skipped period.

These experiments demonstrate that the endometrium, after being under the influence of estrogen for several weeks, becomes very sensitive to withdrawal of the hormone. That such sensitivity depends on the length of treatment is clearly shown by the fact that bleeding does not occur following a 48-hour-skipped period when estrogen has been given for 22 days for the first time. The injection of estrogen was omitted for 2 days in 5 juvenile animals after an initial treatment of 1000 I.U. daily for 22 days, and none had bled by the end of 9 days. These results also indicate that bleeding 22 days previous to a skipped period in an animal used repeatedly in such experiments does not restore a condition in the endometrium physiologically identical with that at the beginning of an initial treatment.

Changes in the sexual skin. An increase in edema and coloration of the sexual skin of castrated rhesus monkeys is a normal response to the injection of estrogen, but the degree and character of the reaction depends on the age of the animal. The sexual skin undergoes a progressive maturation during puberty and adolescence (2). Puberty is heralded by a blister-like enlargement of the pubic lobes. This tends to disappear during adolescence which is characterized by peripheral lobulated swellings. Coloration of the sexual skin begins during adolescence and ordinarily is associated with little or no edema in the adult animal. Response of the sexual skin to estrogen is characteristic of the stage of maturity, when the treatment is begun immediately after castration.

The young animals used in these experiments all showed either swelling of the pubic lobes or some reddening and folding of the surrounding skin, or both, by the end of the first 20 days on estrogen. When progesterone was added to the treatment on the 21st day (1 mg. daily for 1 to 5 days), color and edema of the sexual skin usually decreased noticeably by the 25th day, and marked involution occurred by the 30th to the 35th day, after which the color and some edema gradually returned. Although the animals were continuously under the influence of estrogen, the swelling of the sexual skin following the 1st progesterone treatment was never as extensive as before and after 2 or 3 such treatments usually failed to respond fully, even when the injection of estrogen was continued for several weeks. In contrast with this, the coloration became progressively better developed, giving the sexual skin an adult appearance.

It is surprising to find that 1 mg. of progesterone in the presence of 1000 I.U. of estrogen will initiate wilting of the sexual skin 5 days after it is injected and that the process of involution will continue for another 10 days. A good example of this is outlined in the following protocol.

Monkey Al. 85. Adolescent, wt. 3700 gm. Castrated and given 1000 I.U. of estrogen daily for 20 days. Sexual skin very edematous and moderately colored. One mg. of proges-

terone injected on 21st day, estrogen treatment continued uninterruptedly. Sexual skin showed slight loss of edema and color on 5th day following injections of progesterone. Eighth day, sexual skin markedly reduced. Ninth day, edema nearly gone and color pale. Tenth day, blood in vaginal lavage. Fifteenth day, sexual skin greatly involuted. Seventeenth day, sexual skin becoming bright red and swelling rapidly.

This monkey (*Al. 85*) is the only one in our series that was given only 1 mg. of progesterone at the conclusion of the first 20 days of estrogen treatment. The others that were given this dosage had undergone 1 or more previous treatments, and considerable maturation of the sexual skin had been induced; consequently the swelling was much less than in this experiment and the induced involution correspondingly less striking. Therefore, it cannot be stated with certainty that 1 mg. will always produce such a marked effect, but that it did so in this 1 instance seems indicative and at the same time serves as a typical example of the reaction that invariably occurred when the administration of progesterone was extended for 2 to 5 days.

The fact that a dose of 1 mg. of progesterone will bring about a pronounced loss of edema and blanching of the sexual skin in the presence of estrogen may be of considerable importance in explaining the physiology of the 2 hormones. It also seems significant that the effects of such brief treatments with progesterone should continue for as long as 10 to 15 days. It is improbable that progesterone lingers in the body for so long a time. It seems more likely that progesterone modifies the sexual skin in a way that renders it unresponsive to estrogen and that some 10 days are required to recover the original physiological condition.

The changes in edema and color of the sexual skin in response to estrogen and progesterone may be due to a modification of the vascular and lymphatic supply. Zuckerman (3) is of this opinion, and Collings (4) found a direct correlation between the degree of reddening and the size and degree of engorgement of the superficial vessels. However, the 5-day interval between the injection of a single dose of progesterone and the beginning of a loss in edema, as well as the 10-day recovery period, suggest a conditioning of the tissues in a way similar to that described for the uterus which involves a shift in water balance and electrolyte pattern (5, 6).

That progesterone produces a characteristic effect is further indicated by a difference in the response of the sexual skin following a few injections of progesterone while the animal is receiving estrogen, and skipping 2 days during a chronic estrogen treatment. In 4 juvenile monkeys into which estrogen was not injected on the 21st and 22nd days, the edema of the sexual skin was noticeably reduced by the morning of the 23rd day, and considerable turgidity was regained by the 24th day—about 24 hours after the injections had been resumed. The bright red sexual skin of 2 mature animals on continuous estrogen treatment showed a decrease in color by the 1st day following a skipped period of 2 days; but unfortunately the injections were discontinued, and the length of time required for recovery was not determined. The changes in the edema and perhaps the color of the sexual skin resulting from a 2-day skipped period during a treatment with estrogen are of brief duration as compared with the rather long recovery period following termination of a short series of progesterone injections. The loss of edema and color caused by a withdrawal of estrogen for 2 days is no doubt due to the absence of estrogen; but following the discontinuance of progesterone, the long interval during which the sexual skin does not respond to estrogen suggests that physiological changes have been induced and that the refractory period is a measure of the time required for recovery.

DISCUSSION

There seems to be general agreement that in both monkeys and women menstruation may occur either from a progestational endometrium or from an endometrium

that shows only the effects of estrogen and that bleeding may be precipitated experimentally by withdrawing either estrogen or progesterone. Also, the injection of progesterone for only a few days into monkeys on continuous estrogen treatment causes bleeding (7–10), and apparently the same is true for women (11). The work reported here was undertaken with the idea that a more exact knowledge of the nature of the physiological action of estrogen and progesterone on the endometrium could be had by studying the interaction of the 2 hormones when employed in amounts approaching minimal maintenance doses. A daily dose of 1000 I.U. of estrogen is safely above the threshold required to maintain the endometrium without bleeding during a chronic estrogen treatment (1). None of our animals bled while on this dosage though continued in some instances for over 100 days. We have also found (9) that 1 mg. of progesterone daily is about the minimal amount of this hormone that will inhibit bleeding indefinitely following the discontinuance of a 20-day treatment of about 1000 I.U. of estrogen daily.

Perhaps the most surprising as well as most important point brought out by these experiments is that 1 mg. of progesterone given in 1 dose will precipitate bleeding during a chronic treatment with a maintenance dose of estrogen. In fact 2 of 4 monkeys bled when 0.5 mg. of progesterone was injected intradermally into the sexual skin. The histological changes in the endometrium as a result of such treatment must indeed be very slight, since the only modifications noticed when 1 mg. was given daily for 5 days was a small increase in thickness of the glandular epithelium and perhaps some additional accumulation of glycogen. The fact that such small amount of progesterone can produce bleeding in the presence of estrogen is of especial importance with regard to a possible new viewpoint on the immediate cause of menstruation.

Judging from the amount of sodium pregnanediol glucuronidate excreted in the urine during human menstrual cycles, there must be a wide variation in luteal activity. The total amount excreted during a single cycle may vary from 3.0 to 54.6 mg., and the duration of its excretion may vary from 3 to 12 days (12). Yet regardless of the quantity or the length of time over which sodium pregnanediol glucuronidate is excreted, bleeding occurs from 0 to 3 days following the disappearance of the compound from the urine. It seems more than suggestive that menstruation in all of these instances was occasioned by the withdrawal of progesterone.

The presence of pregnanediol in the urine during the menstrual cycle seems to furnish an adequate measure of the duration of luteal activity, but the amount excreted does not give an exact quantitative estimate of the progesterone secreted by the luteal tissue. Venning and Browne (11, 12) found that the amount of progesterone that could be recovered in the urine as sodium pregnanediol glucuronidate depended in large measure upon the condition of the uterus at the time the hormone was injected. In cases of hysterectomy or atrophic endometrium, the recovery was poor; when progesterone was given during the luteal phase of the menstrual cycle or early pregnancy, the recovery was greater. Yet, the amount of pregnanediol excreted in the urine ranged from 0 to only a fraction of the progesterone injected. Hamblen et al. (13) gave intramuscular injections of 5 mg. of progesterone daily and report that they obtained no evidence that it was converted into pregnanediol. Browne (14) found that menstruation could be induced in cases showing cystic glandular hyperplasia in the endometrium by giving 4 intramuscular injections of 5 mg. of progesterone spaced every other day. Also, Seegar (16) was able to induce endometrial transformation with 10 daily doses of 5 mg. of progesterone without a consistent appearance of pregnanediol in the urine.

These observations indicate that the pregnanediol excreted in the urine may represent only a small part of the total amount of progesterone secreted by a corpus

luteum. They also suggest that progesterone may be secreted in sufficient quantity to induce endometrial modification and bleeding without the appearance of pregnanediol in the urine. In other words, the threshold dose of progesterone required for endometrial stimulation is below that at which the hormone is excreted as pregnanediol.

The fact that bleeding can be brought on by such a brief treatment with progesterone makes it seem unwise to conclude that the absence of ovulation in a menstrual cycle necessarily precludes the possibility of any luteal function and that bleeding is due entirely to a decrease in estrogen secretion. There are many reports in the literature of partially luteinized atretic follicles during the menstrual cycle of women. There is also indirect but nevertheless convincing evidence that in the guinea pig, rat, and rabbit, secretion of progesterone begins prior to ovulation (15, 17, 18). It seems evident that all degrees of luteal function may be found, ranging from that in cycles in which ovulation and full development of a corpus luteum occur to those in which neither ovulation nor luteal formation takes place. Also, it is quite probable that menstruation ordinarily is precipitated by the cessation of progesterone secretion either from a corpus luteum or from an atretic follicle, while bleeding brought on solely as a result of the withdrawal of estrogen might be much less frequent than previously thought.

The modifications in edema and color of the sexual skin brought about by progesterone in the presence of estrogen are interesting because they parallel the changes thought to occur in the endometrium under the same situation (17, 19). First, there is deturgescence, i.e., a loss of water, which is also true of the uterus. Unfortunately the sexual skin of the juvenile rhesus undergoes a process of maturation, so that ordinarily it does not fully regain its original edema once it is reduced by progesterone. One gets the impression that progesterone actually hastens maturity of the sexual skin. This does not seem true of the South African baboon (*Papio porcarius*) which was used by Gillman and Smyth (20) in similar studies. They found that when 3 mg. or more of progesterone is given in a single injection during the 1st or follicular phase of a normal cycle, the relatively enormous perineal swellings, which may attain a width of more than 29 inches, pass rapidly through deturgescence and reach a flabby resting condition within 5 to 7 days and after a delay of about 24 hours once again begin to turgesce. If 20 mg. of progesterone is given, bleeding usually occurs in 5 to 7 days at the end of the induced deturgescence. A single dose of 10 or 15 mg. early or late in the first half of the cycle causes perineal deturgescence without bleeding, while a total of 15 mg., if divided into 2 or 3 injections and given at 3 or 4-day intervals, will bring about both deturgescence and bleeding (21, 22).

The results reported by Gillman for the baboon parallel those given here for the *Macaque* with the exception that the monkey seems much more sensitive to progesterone. However, the baboons were normal animals and were given progesterone during the 1st half of the cycle, while the monkeys were castrated and had received 1000 I.U. of estrogen daily for at least 20 days before being subjected to progesterone. If one takes the latent period between the discontinuance of injections of either estrogen or progesterone and the time of bleeding as a measure of sensitivity, it seems as if the endometrium becomes more sensitive to the withdrawal of either hormone as the treatment is prolonged. When estrogen is discontinued after 1 or 2 weeks, bleeding begins on an average of 9.2 days (23); but if the treatment is continued for several weeks, bleeding may occur within 48 hours after the hormone is withdrawn (24). An increase in sensitivity of the endometrium to a withdrawal of estrogen is also shown by the fact that at the end of a 14-day period of treatment the injections may be discontinued for 4 or 5 days without precipitating bleeding (1), while bleeding occurs in

cases on chronic treatment when deprived of the hormone for only 2 days. Eight of the 10 experiments recorded here gave positive results with bleeding on an average of 2.37 days after the 2-day skipped period with a range of 0 to 4 days. These results are in agreement with an earlier observation (24) that castrated monkeys which had received estrogen for several weeks may bleed within 2 days after discontinuance of the treatment.

The endometrium also becomes progressively sensitized to a withdrawal of progesterone as the treatment is prolonged. A fully sensitized condition can be established in 10 days by giving 0.5 to 1 mg. of progesterone daily following a brief treatment with estrogen. Engle (23) finds that in such experiments bleeding occurs on an average of 2.9 days after the injections of progesterone are discontinued. The results reported here show clearly that a sensitivity of different degrees to the withdrawal of progesterone can be established during a chronic treatment with estrogen. If only those cases are considered in which bleeding was successfully induced, the following comparison can be made between the number of days progesterone was given (1 mg. daily) and the average elapsed time between the last injection of the hormone and the beginning of bleeding: 5 days—3.5 (8 animals); 3 days—4.2 (4 animals); 2 days—5.4 (5 animals); 1 day—7.7 (9 animals). The evidence indicates that, with proper conditioning, the endometrium becomes equally as sensitive to a withdrawal of estrogen as to a withdrawal of progesterone, but with estrogen the process requires several weeks while with progesterone it is a matter of days.

The effects produced in the endometrium by small doses of progesterone during a chronic treatment with estrogen apparently are directly responsible for the precipitation of bleeding. The exact nature of the reaction is at present unknown, but certain aspects have been quite well established. The minimal amount of progesterone (about 1 mg.) that will cause bleeding in the presence of estrogen (1000 I.U. daily) does not produce any conspicuous morphological modification of the endometrium, with the possible exception of a detectable loss of edema. It also seems evident that the effects of a single injection of progesterone last for several days, much longer than one should expect the hormone to remain in the body.

Therefore, it is not the presence of progesterone but the endometrial situation established by progesterone that is responsible for the failure of estrogen to prevent bleeding. The nature of this situation, as far as the effectiveness of estrogen is concerned, seems to be that of raising the threshold for estrogenic stimulation. The conditioning of the endometrium by progesterone is a progressive process related to dosage and time, and the reaction, at least in its early stages, is not irreversible (10).

The idea that progesterone prevents the action of estrogen on the endometrium may be misleading (25). Estrogen in the presence of progesterone is not without effect on the endometrium. In fact, there is a marked synergism both in the inhibition of bleeding (9) and the development of a progestational endometrium (26, 27) when the 2 hormones are administered concurrently. The cooperation of the 2 hormones in this synergistic action is one in which estrogen furthers and augments progesterone in the growth and development of a progestational condition in the endometrium.

Progesterone produces physiological and biochemical changes in the endometrium that in several respects are directly opposite to those brought about and maintained by estrogen (6, 19). Consequently, a physiological situation is established by progesterone which is contrary to the normal action of estrogen. Apparently the endometrium becomes progressively more refractory to estrogen as the progestational reaction proceeds until finally a point is attained at which the original condition, produced by estrogen cannot be restored nor deterioration and bleeding prevented by estrogenic action. Estrogen is not inhibited but serves in a minor rôle as a synergist to proges-

terone, which dominates the reaction and becomes indispensable for the preservation of the endometrium. Consequently, the bleeding that occurs in the presence of estrogen following a brief treatment with progesterone is due to the absence of progesterone. It seems quite probable that this is also true of menstrual cycles in which progesterone is secreted, which may occur in the absence of ovulation and be insufficient in duration and amount to produce conspicuous morphological changes in the endometrium or appear as pregnanediol in the urine.

SUMMARY

Castrated adolescent monkeys (*Macaca mulatta*) on continuous estrogenic treatment with 1000 I.U. of estradiol daily were given, between the 20th and 30th days, 1 I.U. of progesterone daily for periods of 1 to 5 days. Menstruation in the presence of estrogen occurred, on an average, on the following days after the discontinuance of progesterone: 5 days—3.5; 3 days—4.2; 2 days—5.4; 1 day—7.7. The injections of estrogen can be omitted for 2 days at the end of 20 days without precipitating menstruation, but when the treatment has continued for several weeks, bleeding may follow such skipped periods on an average of 2.37 days. This indicates that the endometrium becomes equally as sensitive to a withdrawal of estrogen as to a withdrawal of progesterone, but with estrogen the process requires several weeks, and with progesterone it is a matter of days.

Progesterone under these conditions also produced marked loss of edema of the sexual skin. Maximal deturgescence was attained within about 10 days regardless of whether progesterone was given for 1 or 5 days.

The bearing of these observations on the problem of menstruation is discussed.

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DOES ESTROGEN SUBSTITUTION MATERIALLY INHIBIT PITUITARY GONADOTROPIC POTENCY?[1,2]

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THAT SMALL DOSES of estrogens may exert a profound influence upon the mammalian organism without materially depressing the gonadotropic action of the pituitary gland has been previously reported from this laboratory (1, 2). Our initial interest in the relationship between the gonads and the pituitary gland was aroused by the generally accepted belief that the gonadotropic action of the pituitary gland is entirely regulated by the secretions of the gonads, and more specifically, that the estrogenic hormones exert an inhibitory or depressant effect upon the gonadotropic action of the hypophysis.

When crystalline estradiol was made available to us early in 1937,[4] an attempt was made to quantitate this relationship by treating castrated rats with graduated doses of estradiol, and later evaluating the effect by assaying the pituitary glands from these animals. In this effort we had a number of advantages contributing to the accuracy of the experiment that the earlier workers in the field did not have. Beside the obvious advantage of being able to use exactly measurable amounts of a pure crystalline estrogen, the hazards of pituitary assays by the implantation method were circumvented with the use of a daily injection technic of pooled and macerated glands from a number of rats which contributed to the uniformity of the results. Further, the uterine-weight assay endpoint increased the sensitivity of detection of gonadotropic substances 8 to 10 times over that of the ovarian-weight assay endpoint (3). The results were disappointing in that it did not show a quantitative effect upon pituitary gonadotropic potency, although the estradiol action was exerted in a quantitative direct manner upon the vaginal mucosa, thymus gland and in causing pituitary hypertrophy. In fact, estradiol administration up to 62 R.U. daily for 20 days showed no gonadotropic-depressant action whatever in castrated rats, as indicated in figure 1. The doses used seemed more than adequate in preventing postcastration changes in the vaginal mucosa and in the thymus gland, since the highest dose used (62 R.U. daily) caused constant vaginal estrus, thymus atrophy, and pituitary hypertrophy without altering the postcastration rise of gonadotropic hormone content per whole gland.

Belief in the principle that gonadal hormones suppress pituitary gonadotropic function has led clinicians to use estrogens and androgens when such suppression

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seemed desirable. Moreover, results obtained with the administration of sex hormones to patients in many clinical conditions have been at times indiscriminately attributed to their action in inhibiting pituitary function, often without any objective evidence of such an inhibition.

Taking advantage of the improved methods of concentrating and assaying urinary gonadotropic hormones available (4) we undertook to test the effect estrogens exert clinically upon gonadotropic excretion. The amounts of gonadotropin excreted by normal women approaching the menopause, women in the menopause (both those

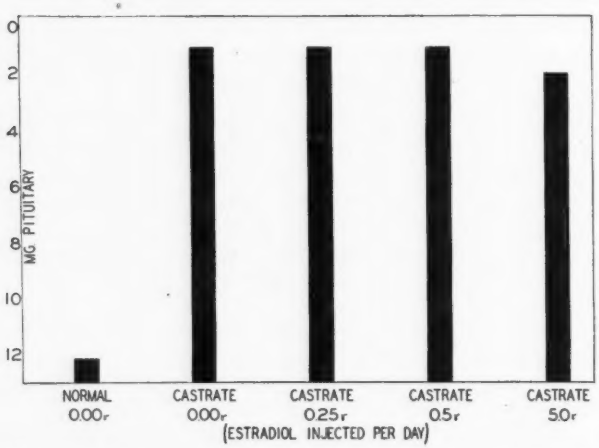

Fig. 1. Gonadotropic potency of pituitary glands of 20-day castrate female rats. Height of each column indicates the amount of fresh pituitary gland in mg. necessary to produce a minimal increase in uterine development in recipient rats. The apparent decrease in potency in the 5.0 μg. estradiol-injected group is due to the considerable increase in pituitary weight. When calculated on a per gland basis all castrate groups, injected and uninjected show approximately the same gonadotropic potency.

having symptoms referable to the menopausal syndrome and those not having these symptoms), and also the excretion by women well past the climacteric were determined. Both natural and artificial menopause was thus standardized as to gonadotropic excretion. It was found that the level of hormone was uniformly high in all these cases except in the women having normal cycles. Then a series of women was tested who initially had active symptoms of the menopause, but whose symptoms were now adequately controlled with small doses of estrogens, ranging from 2000 I.U. given orally every day to 10,000 I.U. given intramuscularly thrice weekly. No material difference was detected in the amount of hormone excreted by the women in the menopause but not treated, and those whose symptoms had been controlled with treatment (5). See figure 2.

These findings in estrogen-treated women corroborate the experimental results obtained previously with the estrogen-treated 'menopausal' or castrated rats. Our results are at such complete variance with the generally accepted principles regarding the action of sex hormones on the pituitary hormones that a further explanation seemed indicated. The most obvious explanation is that in all of the experiments we adhered to the principle of using the minimal dosage of estrogen that will produce the desired effect. In the case of the menopause patients, the endpoint of desired effect was clinical improvement (and more objectively the change to a more active vaginal smear picture); and in the castrated rats, the prevention of castration changes. Use

of larger amounts of estrogen in the treatment of the menopause, and in the castrated rats may conceivably still produce these desired effects, and in addition produce suppression of the pituitary. However, the use of large amounts of estrogen which obviously overstep the bounds of physiological limits in experiments designed to investigate the pituitary-gonadal relationship, is not a rational means of determining how that mechanism functions in normal animals. Therefore in the subsequent experiments undertaken to study this relationship, similar small doses of estrogen were employed.

The possibility that in the above instances the entire absence of functioning ovar-

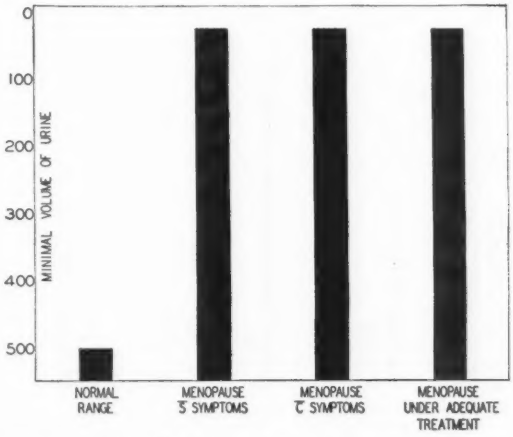

Fig. 2. GONADOTROPIC HORMONE EXCRETION IN MENOPAUSAL WOMEN. Each column represents an average of many urine samples from many women. The height of the columns shows the least amount of original urine, which was concentrated by alcohol precipitation, necessary to give minimal uterine stimulation in assay rats. The column representing the normal includes only those women of menopause age who still have regular cycles, and represents the base level of excretion not the peak excretion rate.

ian tissue might alter the relationship to such an extent that an irreversible change took place, led to the selection of the unilaterally castrated female as the present test animal. In this type of experimental animal, since one ovary remained functional, it was postulated that the rise of gonadotropic potency following removal of the one ovary, if it occurred at all, would not be as great as that following complete castration or following the inception of the menopause. Thus the entire endocrine balance would be maintained close to the normal range and perhaps could be more easily influenced. In addition, the presence of one functioning ovary throughout the procedures could act as an autogenous indicator as to what was happening in the animal body.

Virgin female rats 6 to 8 months of age were utilized as the donors. At the time of unilateral castration, where the right and then the left ovaries were removed in alternating rats, the removed ovaries were weighed so as to serve as auto-controls. The estrogen injections followed immediately for periods of 10 and 20 days respectively, at the end of which time the rats were killed and autopsied. Similarly, unilaterally castrated rats that did not receive injections were killed at the end of the 10 or 20-day period to serve as controls. For still further comparisons, and as additional controls, normal intact animals, some groups of which were injected with the same amounts of estrogens as above and others of which were uninjected, were

autopsied at the end of the 10 and 20-day periods. In order to obviate the criticism that the doses were too small, the relatively high amount of 5 micrograms (62 R.U.) of estradiol was injected in sesame oil daily. A total of 102 donor rats were used to make up these groups.

As could be anticipated, the most marked deviation from the normal intact controls caused by unilateral castration occurred in the ovarian weights (fig. 3). As can be seen in table 1, the maximum weight change had already occurred at the end of 10 days and was not materially greater 20 days after operation. The weight of the remaining ovary was roughly 15 mg. over that of the ovary removed at operation, and of course, it had gained the same amount over the single ovary weights of the intact

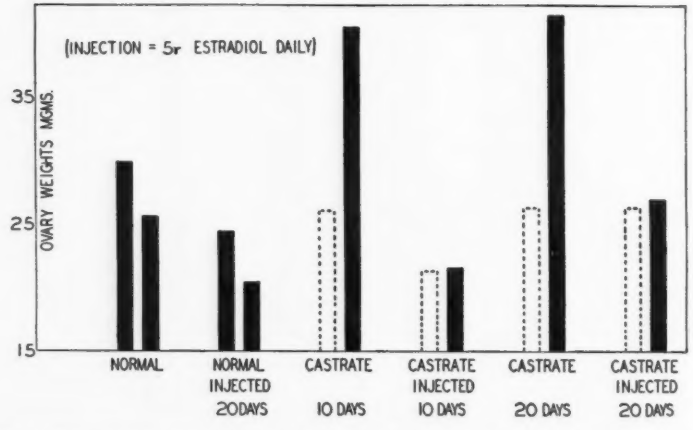

Fig. 3. OVARIAN WEIGHTS IN UNILATERAL CASTRATED FEMALE RATS. Solid columns indicate ovarian weights obtained at autopsy. The dotted columns indicate weights of the ovaries removed at operation 10 or 20 days before autopsy. In the normals, one bar represents right, and the other, left ovarian weights.

normal controls. Unilateral castration produced no significant weight change in either the thymus or pituitary glands as compared to the normal intact controls. By following the vaginal smear picture of the operated rats it was found that normal cycles were resumed within a short time postoperatively. Pituitary gonadotropic potency, as determined by injecting the pooled macerated anterior lobes into 22-day-old recipient rats and following the effect upon the ovarian and uterine weights, was not significantly increased either at the end of the 10 or 20-day period. We have been unable to account for the apparent increased potency of the pituitaries of the 5 rats in *group F*, but have included them in tabulating the average results, which they do not alter significantly. It may be concluded, therefore, that within 10 days following the removal of one ovary in the rat, no significant measurable alteration takes place in the animal except compensatory hypertrophy of the remaining ovary. This compensatory hypertrophy, incidentally, is not great enough to cause the remaining ovary to weigh as much as the 2 ovaries initially.

The effects of injecting 5 micrograms (62 R.U.) of estradiol daily immediately following the removal of one ovary may be compared with findings in the uninjected unilateral castrates. That the estradiol was producing a marked effect was apparent from observing the vaginal smears, which exhibited uniformly a predominance of nucleated epithelial cells. These were atypical and suggested that changes similar to

TABLE 1. RESPONSE OF FEMALE RATS TO 10 AND 20 DAYS OF UNILATERAL OVARIECTOMY AND ESTRADIOL SUBSTITUTION

Group No.	No. of Animals	At Castration		At Autopsy						Recipient Assay[1]		
		Body wt. gm.	Ovary[2] wt. mg.	Body wt. gm.	Ovary wt. mg. Rt.	Ovary wt. mg. Lt.	Ovary wt. mg. Loss-gain	Thymus wt. mg.	Pit. wt. mg.	No. of animals	Ovary wt. mg.	Uterus wt. mg.
intact controls												
A	5	237		248	31.8	24.4		114	10.9	5	12.2	36.5
B	4	262		263	28.0	26.2		103	13.6	4	13.7	59.7
C	15			252	29.9	26.4		131	11.3	12	11.4	47.6
Av.	24			253	29.9	25.9		123	11.6	21	12.0	47.3
10-day castrate controls												
A	5	243	29.8	251		43.3	+13.5	120	13.0	5	11.3	38.3
D	14	241	27.6	241		41.1	+13.5	127	12.6	14	11.3	44.8
F	5	229	21.5	234		36.8	+15.3	133	12.6	5	13.3	122.0
Av.	24	240	26.8	242		40.7		127	12.7	24	11.7	59.2
10-day castrate injected												
A	5	242	22.5	220		17.8	−4.4	54	13.2	5	13.1	52.2
D	14	229	22.8	217		24.8	+2.1	67	12.2	14	13.3	91.0
Av.	19	233	22.6	218		23.0		63	12.4	19	13.3	80.8
20-day castrate controls												
B	5	252	26.7	245		42.6	+15.9	97	14.0	5	14.8	71.9
E	4	248	27.5	248		42.6	+15.1	166	14.5[3]	4	11.2	47.2
Av.	9	250	27.1	246		42.6		128		9	13.2	60.9
20-day castrate injected												
B	5	263	25.2	238		25.7	+0.5	44	16.4	5	11.8	55.8
E	6	238	28.7	229		30.1	+1.4	82	15.6[3]	4	9.2	52.3
Av.	11	249	27.1	233		28.1		64.3		9	10.6	54.2
intact control injected 20 days												
C	15			247	24.4	20.9		60	12.9	10	11.3	42.2

[1] Uterine weights of uninjected 26-day-old recipient assay rats average 20 mg.
[2] At castration either the right or the left ovary was alternately removed.
[3] Included posterior lobe. Injections were 5 micrograms per day. Animals with same group numbers were run at the same time.

pregnancy were occurring. As an explanation of the progesterone-like picture seen in the vaginal smears, it should be noted that the ovaries showed a predominance of corpora lutea over follicles as observed at autopsy.

The compensatory hypertrophy of the remaining ovary which occurred in the uninjected groups was completely prevented by the estradiol injections. In fact in group A of the 10-day-injected castrate rats, the ovarian weights were below those of the normal auto-controls removed at autopsy, and below those of the intact uninjected normals; actually atrophy rather than hypertrophy resulted from estradiol substitution. Associated with the prevention of ovarian compensatory hypertrophy was the pronounced thymus atrophy which resulted. This occurred after both 10 and 20 days of injection, in the unilaterally castrated groups as well as in the intact normal injected rats (fig. 4). Pituitary gland weight was not materially affected by the injections.

Estradiol injections did not inhibit the pituitary gland potency in the unilaterally castrated rats as compared to normal intact or uninjected unilaterally castrated control rats in either 10 or 20 days. The only appreciable deviation from these controls

occurred at the end of the 10-day period, and was not a suppression, but an increase in the potency of the pituitary glands as illustrated in figure 5.

The intact normal controls which were injected with the same amounts of estradiol for the 20-day period differed from the uninjected controls only in the marked thymus atrophy, nucleated cells of the vaginal smears, and questionable atrophy of

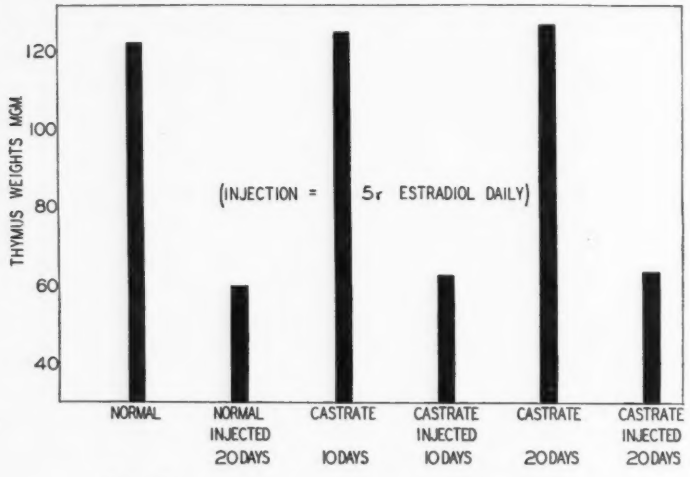

Fig. 4. THYMUS WEIGHTS OF UNILATERALLY CASTRATED FEMALE RATS.

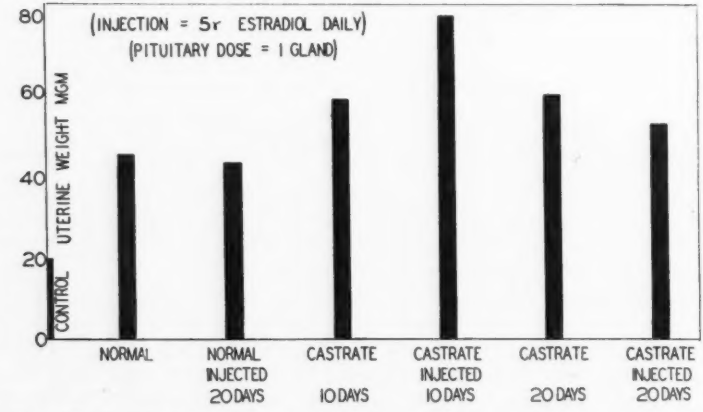

Fig. 5. GONADOTROPIC POTENCY OF PITUITARY GLAND IN UNILATERALLY CASTRATED FEMALE RATS. In each group the anterior lobes of the pituitary glands were pooled, macerated and injected in saline suspension twice daily for 3 days in one-gland-equivalent amounts.

the ovaries. Neither the pituitary weight nor the pituitary potency was affected (in referring to table 1, to make accurate comparisons, compare the groups that were run at the same time and so designated by letter).

Thus small and moderate doses of estradiol circulate in sufficient quantity to exert a powerful and somewhat abnormal or unphysiological effect upon the thymus

gland, as demonstrated by its atrophy; and upon the ovaries, as seen in the failure to develop compensatory hypertrophy. Yet, these unphysiological doses are unable to inhibit the gonadotropic potency of the pituitary gland (fig. 6).

The failure of estradiol to inhibit pituitary potency in intact female rats and in unilaterally castrated female rats confirms our previous observations, *a*) that estradiol fails to inhibit pituitary potency of completely castrated rats, and *b*) that clinically adequate doses of estrogens do not reduce urinary gonadotropic hormone excretion in menopausal and castrate women. These findings leave us somewhat at a loss for an adequate explanation. They do not fit any previously proposed concepts. A new and hitherto unconsidered concept may, of course, presently fit the data. We are

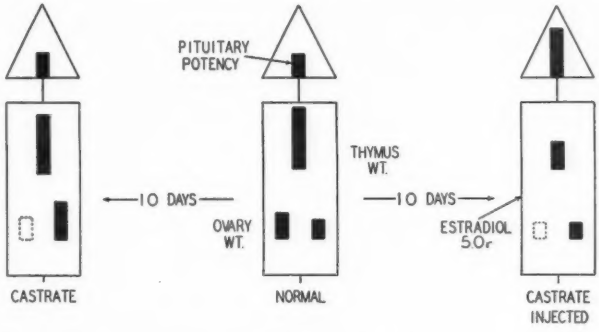

Fig. 6. CHANGES EFFECTED ON OVARIAN AND THYMUS WEIGHTS, AND PITUITARY GONADOTROPIC POTENCY BY A 10-DAY PERIOD OF UNILATERAL CASTRATION AND INJECTION OF 5 μg. OF ESTRADIOL DAILY. The figures for this schematic presentation were taken from the *A group* shown in table 1.

submitting one such concept for consideration, which to date fits all of our data, but not necessarily those of others.

If, as Seidlin suggests (5), the ovary uses up gonadotropic hormone, that is, actually metabolizes it, then the amount of circulating hormone depends upon the amount used by the ovaries. Also the pituitary may be secreting at a fairly constant rate in the adult female. Any increase in blood level and urinary excretion may be due to decreased use, as in the castrate. With production continuing at a constant rate, against an increased blood level, and a peak excretion rate, actual accumulation of hormone in the pituitary gland might occur. This would account for increased gland potency, increased blood level, and increased excretion level of gonadotropins in castrate and menopausal females, as well as in castrate and possibly some climacteric males (7). If the pituitary gonadotropic hormone production level does not provide the control of ovarian activity, what does? Our data fit the concept that ovarian activity is regulated by the blood estrogen level. To illustrate, when one ovary is removed, only half of the estrogen-producing mechanism remains, resulting in decreased blood estrogen levels which stimulate further estrogen production in an effort to return the level to normal. In the course of restoring the blood estrogen level, the remaining ovary hypertrophies. If after removing one ovary, the blood level is maintained by injected estrogen, no increased work is called for, and no hypertrophy results.

Obviously, this theory, to be at all tenable, needs further investigation. At present, all we can conclude is that estrogens in amounts that already overstep the physiological bounds necessary for some organs, do not decrease pituitary potency

in the rat, or gonadotropic excretion in the menopausal female. We do not claim that large doses of estrogens cannot accomplish this,—only that large doses are unphysiological and unnecessary, if not harmful clinically.

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PERCUTANEOUS POTENCY OF ESTERIFIED AND NONESTERIFIED ESTRADIOL

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THE PRESENT STUDY is concerned with the relative percutaneous effectiveness of α-estradiol, α-estradiol benzoate, and α-estradiol dipropionate. The uterine weight of castrated rats after a constant period of treatment was used as the criterion for effectiveness of the estrogens, with the uterine weight of the untreated spayed rat as control. This type of test, measuring a 'graded reaction' was selected in preference to an 'all or none' response type of test, with which a 'minimal effective dose' is determined. Although the latter is the most commonly used method to assay estrogenic potencies (by determining the lowest dose which produces estrus in the vaginal smear of 50 per cent of the animals), we refrained from using it, because this type of test places a premium on rapid absorption. The esterified hormones are, therefore, penalized, because of their slower rate of absorption, and a true indication of their effectiveness relative to the nonesterified forms cannot be obtained. The response of the uterus was studied at 2 dosage levels and with 2 different frequencies of treatment. The relative duration of effect of the 3 estrogens when administered percutaneously was also studied in order to determine whether the superiority of the esterified forms which has been demonstrated parenterally would also be evident by the percutaneous route.

The potency of sex hormones has usually been compared on the basis of equal milligram amounts. In comparisons of the effectiveness of an esterified hormone with that of the nonesterified form, such a procedure penalizes the esterified form, since a certain proportion of its molecular weight is represented by the acid radical, which has no actual hormonal effect. In a milligram of an esterified hormone, there is actually less active substance than in a milligram of the nonesterified form. In a previous paper (1), comparing the action of an esterified and a nonesterified androgen, we have shown that the results obtained when equal active (or molecular) amounts are used are different from those when equal milligram amounts are administered. In the experiments reported in the present paper, the estrogen-containing ointments were made up in such manner that they contained equivalent active amounts of estrogen.

PROCEDURES

Adult female rats, 150 ± 10 gm. in weight, were castrated. Only the ovaries and oviducts were removed, so that the uteri were left intact.

The ointments were applied from a tuberculin syringe to a clipped area (15–20 sq. cm.) on the back of the animal and rubbed into the skin with the barrel of the syringe. By this method of application, a small quantity of the ointment could be accurately

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measured. From the known weight of the hormone content in one gm. of the ointment, and the known weight of 1 cc. of the ointment (0.931 gm.), the dosage was computed. During the treatment period new hair growth was clipped whenever necessary.

In a recent paper, published while our own experiments were far progressed, Miescher and Gasche (2) advised, in studying the effect of ointments containing hormones, the use of a muzzle in order to prevent the rats from licking the ointment. In our experiments, the rats were not observed to lick the anointed area.

In all of the experiments the same type of ointment, an 'oil in water' emulsion was employed. In each instance it was carefully prepared using the same technic. The estrogens were dissolved in sesame oil, and the latter emulsified in water with the aid of protegin X[1] as an emulsifier. Thus, the ointments contained besides the estrogens, 5 per cent sesame oil, 25 per cent protegin X, and 70 per cent water. The concentrations of the estrogens in this ointment base were arranged in amounts proportionate to their molecular weights (estradiol 272, estradiol benzoate 376, estradiol dipropionate 384), and varied according to the needs in the various groups of experiments. The quantities administered are stated hereinafter.

A, *daily treatment; effect on uterine weight.* To determine the effect of daily treatment on the uterine weight, 145 castrated female rats were divided into 7 groups, 1 control and 6 experimental. The number of animals in each group and the doses in micrograms are given in table 1. *Groups I, II* and *III* were treated daily for 21 days

TABLE 1. EFFECT ON UTERINE WEIGHT OF DAILY PERCUTANEOUS TREATMENT WITH VARIOUS ESTROGENS

Group	Substance	No.	Mean Body wt.	Daily Dose	Uterine weight mean and σ_m	Critical Ratio	
						Est. dip.	Est. ben.
			gm.	μg.	mg.		
IV	Estrad.	20	172	1.40	396.5±15.1	0.03	1.27
V	Est. dip.	20	157	1.96	395.8±19.4		1.44
VI	Est. ben.	20	165	1.93	362.8±17.8		
I	Estrad.	25	169	0.7	340.8±12.6	**4.04**	
II	Est. dip.	21	168	0.98	363.7±10.0	1.43	**6.30**
III	Est. ben.	19	168	0.96	280.3± 8.4[1]		
Controls		20	183		97.9± 4.6		

$\sigma_m = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$; C.R. (critical ratio) $= \frac{m_1 - m_2}{\sqrt{\sigma_{m_1}^2 + \sigma_{m_2}^2}}$

Significant critical ratios, in boldface type (2.86 or more with 19 to 25 animals in each group)
[1] $(n-2)$ used in computing for this group.

with 0.05 cc. of an ointment containing 0.015 mg. of estradiol per gm., 0.021 mg. of estradiol dipropionate per gm., 0.0207 mg. of estradiol benzoate per gm. respectively. *Groups IV, V* and *VI* were each treated with 0.1 cc. daily, thus double the amount of the estrogens was administered. Treatment was started the day after castration. On the 22nd day, the animals were killed by cervical fracture, the uteri were carefully removed and immediately weighed.

B, treatment every 3rd day; effect on uterine weight. Sixty-two rats of the same weight, prepared and handled in the same way, were divided into 3 groups (*VII, VIII* and *IX*) to study the effect of the estrogens on the uterine weight when ad-

[1] A commercially available emulsifier (The Goldschmidt Corporation) stated to contain as active ingredients cholesterol and cholesterol related compounds derived from lanolin.

ministered percutaneously only every 3rd day. Instead of 21 administrations, the animals thus received 7 treatments during the 21-day period. Three times the quantity of ointment used in the low-dose-daily groups (*I*, *II* and *III*) was given at each application (0.15 cc.). The total quantity administered during the experimental period, therefore, was the same as in *Groups I*, *II* and *III*.

C, single treatment; duration of estrous effect. Sixty-two castrate animals employed in this series of experiments (*Groups X*, *XI* and *XII*) received 0.2 cc. of the ointment containing 0.05 mg. of estradiol per gm., 0.071 mg. of estradiol dipropionate per gm. and 0.069 mg. of estradiol benzoate per gm., respectively. In the second series, *Groups XIII*, *XIV*, and *XV*, a more concentrated ointment was used, so that a larger dose of the hormone was applied in only 0.1 cc. A control group in this series was, of course, unnecessary. Forty-eight hours after treatment, vaginal smears were made and every day thereafter, until all smears were completely negative. A smear was considered to represent true estrus when leucocytes were completely absent, i.e., the smear was composed entirely of nucleated epithelial cells, or cornified epithelial cells, or combinations of the two. Smears were considered to reflect estrogenic stimulation (but not estrus) when they contained a preponderance of epithelial cells, although leucocytes were present.

RESULTS

A, daily treatment; effect on uterine weight. The data for the 6 daily-treatment groups and the castrated control group are shown in table 1. Comparing the results obtained with the 3 estrogens administered at the low dosage level, it can be seen that the mean uterine weights of *Groups I* and *II* (estradiol and estradiol dipropionate) are not significantly different, while the uteri of *Group III* (estradiol benzoate) are significantly lighter than those of the other 2 groups. At the higher dosage level (*Groups IV*, *V* and *VI*), these differences fade out. Both estradiol and estradiol dipropionate still produced a greater mean uterine weight than did the benzoate, but the deviations were such that the differences have no statistical significance. It will be noted that the dosage increase resulted in a relatively greater rise in the uterine weight in the case of estradiol benzoate (29.4%) than with estradiol (16.3%) or estradiol dipropionate (8.8%).

B, treatment every 3rd day; effect on uterine weight. The data of the 3rd-day treatment groups, *VII*, *VIII* and *IX*, are recorded in table 2. It is evident that all 3 estrogens showed approximately the same potency when administered at 3-day intervals.

TABLE 2. EFFECT ON UTERINE WEIGHT OF PERCUTANEOUS TREATMENT EVERY 3 DAYS WITH ESTROGENS

Group	Substance	No. Rats	Mean Body Wt.	Dose	Uterine Weight mean and σ_m	Critical Ratio				
						3-day-groups		Daily treatment, low dose		
						Est. dip.	Est. ben.	Est.	Est. dip.	Est. ben.
			gm.	μg.	mg.					
VII	Estradiol	21	179	2.09	241.5± 6.7	2.02	0.82	6.96	10.12	3.63
VIII	Estradiol dipropionate	20	173	2.93	269.5±12.1		1.64	4.08	5.98	0.74
IX	Estradiol benzoate	21	175	2.89	248.6± 5.5			6.70	10.05	3.17

See footnotes under table 1.

In spite of the fact that the same total amount was administered as in *Groups I, II* and *III* respectively, the uterine weight was definitely less in those animals which were treated only every 3rd day.

The frequency of treatment, then, modified the effectiveness of these estrogens, but the degree of modification varied with the different estrogens. Estradiol and estradiol dipropionate showed relatively greater loss in effectiveness with less frequent treatment than did estradiol benzoate. Estradiol when given every 3 days lost 29 per cent of the effectiveness it possessed with daily administration. Estradiol dipropionate lost 25 per cent while estradiol benzoate lost only 11 per cent. The smaller loss in effectiveness for the benzoic acid ester accounts for the fact that there were no statistically significant differences among the 3 estrogens when treatment was given at longer intervals, whereas both estradiol and the dipropionic ester were significantly more potent than the benzoic ester when treatment was given daily.

C, *single treatment; duration of estrous effect.* Table 3 shows the average duration of estrus, as well as the average duration of what has been called 'estrogenic stimulation,' caused by the 3 different estrogens at 2 different dosage levels. The durations of effect showed the same order by both criteria at both dosage levels. The true estrus,

TABLE 3. COMPARATIVE DURATION OF ESTRUS AND ESTROGENIC EFFECT FOLLOWING PERCUTANEOUS TREATMENT WITH ESTROGENS.

Substance	Dose	No. Rats	Av. Duration of Estrus	Critical Ratio		Av. Duration Est. Stim.	Critical Ratio	
				Estrad. diprop.	Estradiol benzoate		Estrad. diprop.	Estradiol benzoate
	μg.		*days*			*days*		
Estradiol	9.31	10	1.0±0.47	1.21	1.47	1.8±0.14	**3.24**	**5.74**
Estradiol dipropionate	13.22	11	1.6±0.22		1.29	3.0±0.35		1.72
Estradiol benzoate	12.85	11	1.7±0.14			3.8±0.32		
Estradiol	46.55	10	2.5±0.53	2.53	**4.08**	5.1±0.39	2.73	**4.81**
Estradiol dipropionate	66.10	10	4.3±0.39		1.45	6.9±0.54		1.21
Estradiol benzoate	64.24	10	5.2±0.48			7.7±0.39		

Significant critical ratios in bold face type; (3.00 or more with 10 to 11 animals in each group).

$$\sigma_m = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$$

as well as the estrogenic stimulation, persisted longer with the esterified estrogens than with the nonesterified estradiol. With both dosages, estradiol produced the shortest duration of effect, while the effectiveness of the dipropionate ester was in between, but closer to that of the benzoate than to the nonesterified substance. The difference in duration of estrogenic stimulation produced by estradiol and by the benzoate was significant in both dosage groups, but the difference between these 2 in duration of true estrus was significant only with the higher dosage. The superiority of estradiol dipropionate over the nonesterified estradiol was apparent with both criteria (true estrus and estrogenic stimulation); however only at the lower dosage was the critical ratio of the difference above 3.0, which was considered the limit for a significant difference with the number of animals used in this experiment and this also only with regard to the estrogenic stimulation and not with regard to true estrus.

In spite of the fact that the order of effectiveness found with the 2 esters was always consistent with respect to the actual average values, the differences between

them were not significant. It is worth noting that the order of effectiveness of these two esters changes when the substances are given percutaneously as compared with the subcutaneous route. While in the latter instance, estradiol dipropionate has the longer duration of effect, it loses this superiority over the benzoate with percutaneous administration. It may also be mentioned that the degree of prolonged action of the esterified estrogens compared to that of the free estrogens was in no instance as striking with percutaneous administration as has been reported for subcutaneous administration (3–6).

COMMENTS

Our results demonstrate that one of the factors influencing the effectiveness of parenterally administered estrogens, namely the frequency of treatment, also modifies the effectiveness of the percutaneously administered estrogens. A proper quantity of estrogen, regardless of the form of administration, causes a greater effect if it is subdivided and given at frequent intervals in small individual doses, than if the same quantity is administered less frequently and in larger individual doses.

A second similarity in the effect of estrogens when given by either the parenteral or percutaneous route is the more prolonged effect of the esterified forms. This observation helps explain certain findings in the experiments in which potency was measured by uterine weight. It is generally assumed, for lack of more certain information on the subject, that the prolonged effectiveness of the esters is due to a slower rate of absorption from the site of injection. A similar assumption may also apply to the percutaneously administered estrogens, i.e., the esterified hormones are also more slowly absorbed from the skin. The depot in this instance may be in the stratum corneum of the skin, as it is known that percutaneously applied sesame oil can be demonstrated in this region 7 days after application (7).

Assuming such delayed absorption from a depot, there is a tentative explanation for the incongruent results obtained with daily and less frequent administrations. A quantity of a more slowly absorbed substance, like estradiol benzoate, would be expected to maintain its effect during the interval between treatments, while the effect of the same quantity of a substance which is more rapidly absorbed, for instance the nonesterified estradiol, may have disappeared before the next treatment is given. Such a possibility is supported by the finding that estradiol benzoate lost less of its effectiveness than did estradiol when treatment was given only every 3rd day. With less frequent treatment, the slower rate of absorption acts to the advantage of estradiol benzoate.

Unfortunately, however, these speculations do not completely explain the behavior of estradiol dipropionate in our experiments. This substance also showed a more prolonged period of effectiveness than did estradiol after a single administration. If it, too, were more slowly absorbed, one would expect it to lose less effectiveness than estradiol when the interval between treatments is lengthened. This actually was the case (table 2), but the difference was very small and did not alter the relative potencies of the two. With daily treatment, the dipropionic ester was 6.7 per cent more effective than the nonesterified estradiol and, with treatment every 3rd day, it was 11.58 per cent more effective. However, with both frequencies of treatment, the differences between the dipropionic ester and estradiol were not significant.

Furthermore, attention must be drawn to the fact that, with daily treatment at the lower dosage level, estradiol and estradiol dipropionate were both significantly superior to estradiol benzoate; when the dose was doubled, this superiority disappeared. The effectiveness of each hormone with the larger dosage was increased,

but that of estradiol benzoate was increased proportionately more than that of estradiol and estradiol dipropionate, so that the latter substances were no longer significantly more effective than the benzoate.

It is important, however, to note that the findings presented in this report concern the behavior of these 3 estrogens in this particular vehicle. It is quite possible that the results and the relationship between the estrogens might be different in another vehicle, or in another type of ointment. In other experiments dealing with the percutaneous effectiveness of androgens (8), we have found that the type of ointment used may vary the actual and relative potencies of different androgens. It is not known whether this is true also in the case of estrogens. Since there is no evidence to the contrary, the results obtained here can be considered to apply only when these estrogens are administered in this particular ointment under the conditions described.

CONCLUSIONS

Estradiol and estradiol dipropionate, when administered daily were percutaneously more effective than estradiol benzoate. Estradiol and the dipropionic ester had approximately the same effectiveness. At a higher dosage level, the 3 estrogens retained the same order of effectiveness, but the differences between them were not significant.

When the estrogens were administered every 3rd day, their effects were not significantly different. Each was significantly less effective when given every 3rd day than when given daily. Estradiol benzoate, however, retained more of its effectiveness than did estradiol or estradiol dipropionate.

The effect with a single administration, as measured by the vaginal smear, lasted longer when the esterified estrogens were used, although the differences between estradiol dipropionate and the nonesterified estradiol proved to be not significant. In all instances, estradiol benzoate produced a longer effect, though the differences were significant only in certain instances as discussed.

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THE EFFECT OF HYPOPHYSIAL LESIONS ON MATING BEHAVIOR IN FEMALE GUINEA PIGS[1]

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LESIONS THAT ARE PROPERLY PLACED in the hypothalamus of female guinea pigs may be followed by sterility due to an abolition of mating activities. This sterile condition may or may not be associated with changes in the reproductive organs (1, 2). The majority of the animals show normal ovarian cyclic changes following the operation which indicates that in these the loss of mating activity is not due to a disturbance of hypophysial function. Moreover, properly timed injections of ovarian hormones, of hypophysial extracts and of mixtures of ovarian hormones and hypophysial extracts fail to induce estrous behavior in these animals, although they do so in normal animals (3). On the basis of these experiments it has been concluded that, although the genital changes that may follow hypothalamic lesions are due to a disturbance of hypophysial function, the loss of mating activity is due to a destruction of a part of the central nervous system that is essential for the integration of this complex behavioral pattern.

Dempsey (4) was unable to induce estrous behavior with ovarian hormones in completely hypophysectomized guinea pigs although positive responses were obtained with the animals in which the hypophysectomy was incomplete. This suggests that the hypophysis may play a rôle in regulating mating activities other than that of controlling the output of ovarian hormones, that is, the gonadotropic hormones or some other hypophysial secretion may act synergetically with the ovarian hormones in producing the estrous state. The hypothalamic lesions that abolished mating activities in our series of animals were usually confined to the nervous system proper and seldom extended to the hypophysis. However, it is possible that electrolytic changes may have occurred in the parenchyma of the anterior lobe of the pituitary that could not be detected by the usual histologic methods (2). Such changes might have been responsible for the loss of mating behavior and also for the genital changes that occurred in many of the animals. The experiments to be reported in this paper, therefore, were undertaken in order to determine the effect of direct hypophysial lesions upon the mating activities and genital functioning of female guinea pigs.

METHOD

Five mature female guinea pigs were spayed and brought into heat at least once by the injection of appropriate dosages of ovarian hormones (5). Lesions were then placed in the hypophysis by means of the Horsley-Clarke stereotaxic instrument. The lesions were aseptically placed and the technique utilized was similar to that employed by us in making hypothalamic lesions (1). In one animal 3 punctures were made: one

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in the midline and one at a distance of 1 mm. to either side of the midline. In each case the electrode was lowered through the brain and hypophysis until the base of the skull was reached, then lifted ½ mm. and a lesion was made by passing a current of 3 ma. for 30 seconds. The lesion in this animal, therefore, corresponded in size to the hypothalamic lesions of our earlier experiments. In the other 4 animals, 6 lesions (2 rows of 3 lesions each) were made in a similar manner. The second row of lesions was 1 mm. caudal to the first. These spayed animals were later injected with ovarian hormones to induce mating reactions.

Lesions of varying sizes, dependent upon the number of punctures (1, 2, 3 or 6 punctures), were placed in the hypophyses of 14 normal female guinea pigs in a manner similar to that utilized in making lesions in animals of the preceding group. Following the operation the animals were placed in cages, 6 animals to a cage, and the vaginal membranes were observed daily for at least 2 months. A male animal was then placed with each group of females and daily observations were made. At frequent intervals the males were shifted from one cage to another or else replaced by fresh males. In 2 of the females, the vaginal membranes remained constantly closed, and in these observations were made for mating behavior following the injection of ovarian hormones.

At the termination of the experiment, the animals were killed with nembutal and the brains with the hypophyses attached were removed and placed in formalin. The ovaries of a number of the animals that showed genital disturbances were dissected out and placed in formol-Zenker fluid. The brains were embedded in nitrocellulose and serial sections 30μ in thickness were cut; every fourth section was stained by the Weil method and each next following section was stained with cresyl violet. The ovaries were embedded in paraffin, cut serially at 10μ and stained with hematoxylin and eosin.

RESULTS

The 5 spayed animals with hypophysial lesions were allowed 2 to 7 days for recovery from the effects of the operation and then were injected with 2 courses of ovarian hormones, the second course following the first after an interval of rest. Three of the animals gave good estrous reflexes in response to manual stimulation and they copulated during both trials. The responses were normal both in intensity and in duration. The other 2 animals, both of which had lesions consisting of 6 punctures each, were not responsive during one of the trials but gave good responses during the other.

The brains of the 5 animals were prepared for histological study. The lesions proved to be well placed and in every instance considerable damage was done to the parenchymal tissue of the anterior lobe. In almost every animal the lesion had spread dorsally and destroyed the ventral surface of the overlying mammillary complex. It is estimated that the lesions of the animals with 6 punctures destroyed between ½ and ⅔ of the parenchymal tissue of the anterior lobe of the hypophysis. No significant difference could be noted between the lesions of the animals with negative responses during the one trial and those of the animals that were positive during both trials.

Of the 14 unspayed animals, 3 had only one lesion each. One of these ran regular cycles following the operation while the other two animals ran cycles that were irregular in that the vaginal membranes remained open for intervals that were longer than normal. All 3 of the animals became pregnant after they were placed with males. Parturition was normal.

One of the 4 females with two lesions in the hypophysis ran irregular cycles following the operation. The cycles of the other 3 were normal. Each of the 4 animals became pregnant and delivered normally after they were placed with males.

There were 3 animals each of which had 3 lesions in the hypophysis. Two of these animals ran regular cycles following the operation, while the vaginal membrane of the third remained constantly closed. The latter animal was injected at 2 different intervals with ovarian hormones and positive estrous responses and copulation were observed during both trials. The 2 cyclic animals were placed with males. One became pregnant and delivered normally; the second failed to become pregnant although it was kept with a male for a number of months.

There were 4 animals each of which had 6 lesions in the hypophysis. Three of these animals ran regular cycles following the operation while the vaginal membrane of the fourth remained constantly closed. The latter animal was injected with ovarian hormones at 2 different intervals but failed to show mating behavior during either trial. The 3 animals that ran regular cycles were placed with males but only 1 became pregnant although they were kept with the males for a number of months.

The hypophysial lesions with one exception have been studied. Again, the lesions proved to be well placed and they caused variable amounts of damage to the hypophysis and the overlying mammillary complex. The amount of destruction to the hypophysis varied to some extent with the number of punctures made in producing the lesions. It is estimated that the large lesions destroyed from ½ to ⅔ or more of the hypophysis.

No significant difference could be detected between the lesions of the 4 animals that showed an absence of mating activities and the lesions of the 10 animals that gave positive responses. The 4 unresponsive animals were of the group with the larger lesions (1 animal had a lesion of 3 punctures; the others had lesions of 6 punctures each); however this does not necessarily indicate that the sterility was related to the more extensive damage to the hypophysis since the animal of the series that had by far the greatest destruction to the pituitary became pregnant and delivered normally after being placed with males. The sterility of this group, on the other hand, might be related to the fact that with the larger number of needle punctures, an increasing amount of damage was inflicted upon the more dorsally lying structures.

Five of the 14 operated animals developed genital disturbances following the operation. In 2 animals the vaginal membranes remained constantly closed. The ovaries resembled those of an hypophysectomized animal. The 3 remaining animals ran cycles that were irregular in length. The ovaries of these contained many follicles and the disturbance appeared to be due to a delay in the time of rupture of the mature follicles.

One would suspect that the genital changes would occur in the group of animals with the more severe damage to the hypophysis. This was not the case. As we have mentioned before, the animal in which the most extensive hypophysial damage had occurred had regular ovarian cycles and became pregnant. On the other hand, one of the animals that ceased running estrous cycles suffered but slight damage to the hypophysis.

DISCUSSION

These experiments validate our previous conclusions concerning the absence of mating behavior in the guinea pig following hypothalamic lesions. There can be no doubt but that the amount of damage to the hypophysis of the animals of this series was much greater than any which might have resulted from electrolysis in the hypophyses of the animals of our previous series with hypothalamic lesions. We conclude, therefore, that the absence of mating behavior following hypothalamic lesions is due to the destruction of some mechanism within the hypothalamus which is essential for this behavior, and not to a disturbance of hypophysial function.

Since the beginning of this study, Astwood and Dempsey (6) have reported that estrous behavior can be induced in completely hypophysectomized rats by the injection of ovarian hormones. Their findings are in agreement with our results.

SUMMARY

Lesions of various sizes were placed directly in the hypophysis of 5 spayed female guinea pigs. Following the operation estrous behavior was induced in all of the animals by the injection of ovarian hormones. Three of the animals gave positive response during each of 2 trials while the 2 remaining animals gave positive responses during only one of the trials.

Similar hypophysial lesions failed to abolish mating activities in 10 of 14 unspayed females. Nine of the 14 animals became pregnant after being placed with males, while the tenth, an animal with an endocrine disturbance, showed estrous behavior following the injection of ovarian hormones.

Nine of the 14 unspayed females with hypophysial lesions ran regular ovarian cycles following the operation; 2 ceased running cycles at all, that is, the vaginal membrane remained constantly closed. The 3 remaining animals ran cycles in which the period of opening of the vaginal membrane was greatly prolonged. The hypophysial dysfunction in these animals was not related to the extent of the damage to the hypophysis.

It is concluded that the absence of mating behavior following hypothalamic lesions is due to the destruction of some mechanism within the hypothalamus which is essential for this behavior and not to a disturbance of hypophysial function.

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INDUCTION OF MATING BEHAVIOR IN MALE AND FEMALE CHICKS FOLLOWING INJECTION OF SEX HORMONES[1]

INCLUDING NOTATIONS ON BODY WEIGHT AND COMB GROWTH

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THE USE OF gonadotropic and gonadal hormones to produce some of the elements of adult behavior in male chicks has been reported by several earlier workers. Domm and Van Dyke (1) and Domm (2) observed that male chicks began to crow at 9 days of age following 6 daily injections of pituitary gonadotropin. Attempts at treading appeared in these birds at 13 days, after 10 daily injections. Hamilton (3) injected male chicks with 0.5 mg. of testosterone propionate daily for 27 days, starting from the second day after hatching. The birds first crowed 180 hours after the initial injection, and later showed cock-like fighting and 'wing-flapping.' However, Hamilton did not report treading or other signs of copulatory aggressiveness on the part of the injected males. Horn (4) injected testosterone propionate into young chicks and obtained behavior identical to that described by Hamilton (3). Breneman (5) reported crowing in male chicks 5 days old after the injection of either testosterone propionate or dihydro-androsterone benzoate.

Noble and Wurm (6) induced copulation in an immature Black-crowned Night Heron following testosterone propionate injections. In the male rat, Stone (7) reported that by daily injections of testosterone propionate on and after the ages of 22 to 26 days, the median age of first copulation was set ahead of that of controls by about 20 days.

One purpose of the present investigation was to determine whether the complete copulatory pattern of the adult cock can be induced in male chicks injected with testosterone propionate. The findings of Hamilton (3), of Breneman (5) and of Horn (4) do not prove that such behavior can be elicited in immature androgen-injected birds. Although pituitary gonadotropin produces attempts at treading, the complete mating pattern has never been reported. A second objective of this work was to attempt to produce sexual receptivity and the accompanying 'squatting' behavior characteristic of adult hens, in estrogen-injected female chicks.

MATERIALS AND METHODS

All of the chicks employed in this work were single-comb White Leghorns, sexed at hatching and received at our laboratory when 2 days of age. A total of 63 chicks was divided into two experimental series and studied at different dates; the birds used in *Experiment I* were taken from a different batch than those used in the later work. For all hormone injections the vehicle used was 0.1 cc. of sesame oil.

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[2] This paper represents, in part, material submitted by the junior author in partial fulfillment of the requirements for the degree of Master of Science, New York University.

Experiment I

Group A. This group consisted of 12 15-day-old male chicks. Six males received 27 daily intramuscular injections of 0.5 mg. of testosterone propionate.[3] The other 6 chicks were kept as controls and received daily injections of 0.1 cc. of pure sesame oil. All injections were made into the breast muscles, the side and site of injection being varied systematically from day to day.

Group B. Twelve female chicks, 15 days old, were divided equally into experimental and control groups. Six experimental chicks received 27 daily injections of 0.17 mg. of estradiol benzoate,[3] while the controls received 0.1 cc. of sesame oil. The injections were carried out in a manner similar to that described for the males.

The weights of all chicks were recorded on the day of the first injection, 24 hours after the last injection, and again 37 days later.

Experiment II

Group A. Ten 2-day-old male chicks received daily injections of 0.5 mg. of testosterone propionate for 34 days. Nine chicks of the same age were employed as controls and were injected with 0.1 cc. of pure sesame oil daily.

Group B. Four 2-day-old male chicks were removed from the flock and each was raised in complete isolation. The isolated birds received daily injections of 0.5 mg. of testosterone propionate for 34 days.

Group C. Ten 2-day-old females received 31 daily injections of 0.17 mg. of estradiol benzoate, over a period of 34 days. Five other females of the same age were given the same number of injections of 0.08 mg. of estradiol benzoate. Eleven control pullets were injected with pure sesame oil. No injections were given to the experimental and control chicks in this group on the 20th, 22nd, and 24th days of the experiment.

For the first week all the chicks in *Experiment II* received subcutaneous injections of the hormones. Starting on the 8th day all injections were given intramuscularly, in order to reduce the possibility of leakage from the site of injection. The weights of all of the birds in this experiment were recorded weekly, as were the comb sizes of all of the males. The weights and comb sizes of all males were taken again 3 weeks after the last injection. Throughout this work an effort was made to maintain, as far as was possible, similar conditions of food supply, heat, light, crowding, and hygiene, for experimental and control birds.

Methods of Observation and Testing

Experiment I. Observations for behavior were made daily for about 2½ hours. During this period all the birds were kept in a large runway, lighted from the inside and constructed so that they would not be subjected to outside disturbances. After the first attempts at treading were seen each male was tested in an individual cage with a deeply anesthetized chick, and observations of sexual behavior were recorded. A dead chick was found to suit the purpose just as well, and was later substituted for the anesthetized bird. On the 16th day of the experiment each experimental male was paired with an injected female and placed in an individual cage. The cages were arranged so that the occupants of all could be seen at one time. The male-female pairs were then observed for about 2½ hours daily, and copulations and other sexual behavior were recorded. Frequent substitutions of control females for injected ones were made so that all of the pullets were under observation while paired with males. *Experiment II.* The same general plan employed in *Experiment I* was used in observing and testing the chicks of this series. The isolated birds were observed for crowing behavior for a maximum of 1 hour daily and were later tested for treading after all the injected males of *group A* had exhibited this reaction. Both control and experimental females of *group C* were paired with the injected males at some time during the course of the experiment. The pairs in this experiment were observed for a minimum of 4 hours daily.

Normal Mating Behavior

Copulation in the domestic fowl is generally preceded by a brief courtship in which the male unfolds one wing and skips with little mincing steps in an arc around the female. Skard

[3] Testosterone propionate (Oreton) and estradiol benzoate (Progynon-B) were supplied by the Schering Corporation, Bloomfield, New Jersey.

(8) refers to this behavior as "dancing," while Murchison (9) calls it the "sex invitation." It is the latter term which we shall use. A description of the male's copulatory behavior is given by Skard (8).

The overt sexual behavior of the female is much more passive than that of the cock, and as a result is more difficult to describe. Sometimes the hen will squat spontaneously at the approach of a male, or in answer to his sex invitation. This squatting can be distinguished from the normal crouching of the bird by the fact that in the former case the front part of the body is held lower than the back part, and the tail is raised. Squatting in reply to the cock's clear-cut sexual aggressiveness is the more general case, however. When the male seizes the neck feathers of the female or mounts with one or both feet, the hen may crouch submissively and raise her tail feathers up and to one side so that the contact of the ani can be made. Sometimes the male forces the female down before she submits. It is significant that the number of the male's copulatory attempts always exceeds the number of consummated copulations (8).

As far as we have been able to determine, attempts at copulation do not normally appear until about 4½ months of age. Hens will not submit to copulation until a little later than this; occasionally not until after the first egg-laying.

EXPERIMENTAL RESULTS

Experiment I

Behavior of males. All injected males were first observed to crow on the 4th day, following 3 daily injections of testosterone propionate. At this time crowing occurred infrequently and was of extremely short duration. Hamilton (3) gives an excellent description of the juvenile crow of the chicks, as well as the posture which accompanies it. After 6 injections the chicks exhibited the vicious cock-like fighting and frequent 'wing-flapping' also mentioned by Hamilton. Control males seldom pecked at each other and showed no fighting or crowing.

On the 8th day of the experiment 2 injected birds displayed behavior resembling weak attempts at treading. On the following day, after 8 injections, all of the experimental males exhibited this reaction. Raising of the male's neck feathers when approaching a female, and pecking at her head characterized this activity. Some of the males were also seen attempting to mount a sleeping or crouching female, but such attempts were never vigorously prosecuted.

After 9 injections 4 of the 6 experimental males showed the adult copulatory pattern in treading an anesthetized chick. Except for the absence of the sex invitation and the actual anal contact, this behavior did not differ from that of the mature cock. By the following day all 6 injected birds treaded an anesthetized chick. In most cases the response to the prostrate bird was almost immediate, and numerous treadings of this kind were recorded. Occasionally, it was observed, some of the chicks mounted obliquely or gripped the tail or wing feathers and executed all the copulatory movements although not correctly mounted on the reclining bird. This behavior, however, was uncommon and generally not exhibited in later tests. Attempts to mount live birds were always oriented in the normal fashion. The sex invitation was first observed on the 11th day; it was directed toward a dead chick. Throughout the experiment this preliminary courtship was seen only rarely. After the injected males had been paired with females for several days, the copulatory response to a dead or anesthetized bird was not elicited as frequently or as quickly as was the case in earlier tests.

In addition to treading, another indication of the cock-like behavior of the injected male chicks was revealed by a type of activity less obviously sexual. The males very often called the females to food before eating, or scratched the ground to find food and then held it in their beaks while calling to the female, simulating in this way the behavior of the adult.

Behavior of females. The first normal squatting behavior by an injected female was observed on the 16th experimental day, following 17 injections of estradiol benzoate. By the 26th day all of the experimental females had been seen to squat for a treading male at least once. At the conclusion of the experiment a total of 31 treadings had been observed in which both the male and female exhibited typically adult behavior. Individual differences were apparent. One female was observed to squat 17 times, whereas in another this behavior was seen only once. The other 4 injected females exhibited the squatting reaction, 2, 2, 3, and 6 times during the period of observation. Two of the 6 birds were particularly passive and were seen to squat as soon as the males with which they were paired attempted copulation. None of the control females permitted treading when paired with the injected males.

Body weights. The chicks injected with testosterone propionate were slightly stunted in growth during the course of the injections. During the period of treatment the controls gained an average of 60.9 gm. more than the experimental chicks. The

Fig. 1. Enlargement of a frame from a 16 mm. motion picture film showing a 3-week-old male treated with testosterone propionate treading a dead chick.

difference between the growth of experimental and control birds was also apparent more than a month after the cessation of injections, although during that time the controls gained an average of only 29.1 gm. more than the injected males.

In contrast to the males, the injected females gained an average of 79.1 gm. more than the controls during the course of estrogen treatment. This difference in growth rate disappeared however, after the injections were stopped. In the 37 days following the last administration of hormone, the control females gained an average of 60.2 gm. more than the experimental birds, although the latter group remained, on the average, slightly heavier.

Experiment II

Behavior of males. Seven of the injected chicks crowed less than 40 hours after receiving the first injection, when they were not yet 4 days old. They had received 1 mg. of testosterone propionate. On the following day all injected males except one of the isolated birds were heard crowing. This latter bird was first seen to crow on the 9th day of the experiment. Since the isolated chicks were under observation for only 1 hour each day it is possible that this bird crowed when no observer was present.

Treading of a dead chick first occurred on the 13th day of the experiment. As in *Experiment I* this behavior was characterized by the execution of all the movements exhibited by the adult cock during copulation. After pairing with females the males showed persistent copulatory attempts but the pullets were never observed to squat. Very often a male would mount a struggling female and execute all the copulatory

movements while being carried around the cage on her back. While no extensive records of attempts at copulation were kept, the following figures will give some idea of the strength of the male's tendency to mate. In 2 hours of observation one male made 23 attempts at treading. In 6 cases the male had seized the neck feathers of the female and had mounted with both feet, only to be thrown from her back as she fled. As was the case with the males in *Experiment I* the sex invitation was rarely exhibited by these chicks.

The birds raised in isolation not only crowed and treaded as early as the males of *group A*, but also showed other behavior peculiar to themselves. After less than 2 weeks of isolation it was observed that when the experimenter's hand was put into the cage of one of these birds, the chick would peck at it viciously and very often fight it as if he were fighting with another chick. This activity was in marked contrast to the behavior of the birds raised together who fled at the approach of the hand. On the 24th day of the experiment one of the isolated males extended the sex invitation to the hand of the observer, then mounted, gripped the skin tightly and went through all the movements of the copulatory act. The other isolated birds subsequently showed this behavior, its appearance being easily elicited when the observer put his hand into their cages.

Behavior of females. The injected pullets of this experiment did not exhibit the adult sexual behavior which was expected because of the findings in the earlier study. Although they were under observation for much longer periods of time each day than were the birds in *Experiment I*, none of the chicks was ever seen to squat for a treading male. No difference in behavior between the injected and control females could be discerned. The numerous treading attempts of the injected males, and the partially denuded necks of the females where they had been seized, gave ample evidence of the male's sex drive, but no indication of female sexual behavior was ever observed.

Body weights. During the latter part of the experiment one injected male and 4 females receiving 0.17 mg. of estrogen were observed to be suffering from coccidiosis. These birds were removed from the experiment to prevent the spreading of the disease, and their weights are not included in the data given here. As in *Experiment I*, the testosterone propionate-injected males weighed less than the controls. By the end of the injection period the control chicks had gained an average of 57.6 gm. more than the experimental birds, and in the 3-week period after the cessation of injections they gained an average of 53.1 gm. more than the treated individuals.

The females which received daily injections of 0.17 mg. of estradiol benzoate gained an average of 40.9 gm. less than the controls, from the first injection to the end of treatment. There was no significant difference in weight between the controls and the females injected with only 0.08 mg. of the hormone daily. These data differ from those obtained in *Experiment I* in which the average gain in weight of the injected pullets was greater than that of controls.

Comb growth. Within a few days after the first injection of testosterone propionate there was a noticeable growth of the experimental males' combs. They soon became large, turgid, and extremely thick throughout, and in about 2 weeks had assumed a deep red hue. There was a comparable growth of the wattles, and the earlobes also were larger and more deeply colored than those of the controls. When injections were stopped there was a rapid regression of the combs, so that 3 weeks after the last injection the combs of the controls were considerably larger than those of the experimental chicks. Figure 2 illustrates the comb growth of all male chicks during the course of this experiment.

There was no discernible difference in size between the combs of injected females and those of the controls.

DISCUSSION

Behavior

Males. The psycho-sexual changes accompanying puberty represent the maturation of some of the most complex of the group of delayed instincts. Mating, for example, is an activity that generally requires the execution of complicated, coordinated movements by the male, and which appears, in lower vertebrates at least, fully integrated at maturity without previous practice or learning. That mating behavior in the male fowl is dependent upon the active secretion of testicular hormone is indicated

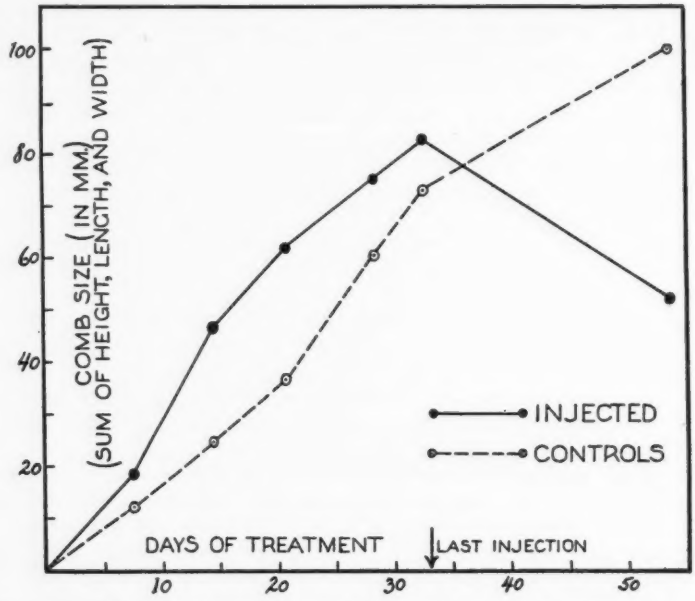

Fig. 2. Comb growth in control and androgen-injected chicks.

by experiments on castration, implantation, and injection. Reviews of the literature on these subjects can be found in references 10 and 11.

In our experiments the behavior pattern involved in copulation by the adult cock was induced in its entirety in immature male chicks after the injection of testosterone propionate. This activity was manifested long before somatic maturity was reached, and antedated its normal appearance by several months. Treading a dead or anesthetized bird may be peculiar to those birds receiving heavy doses of male hormone, since testing a normal sexually-active cock in this manner did not elicit the copulatory response. It is significant, however, that after the injected males had been paired with females for a short time the dead chicks did not serve as adequate stimuli for eliciting copulatory behavior as often as they did earlier in the experiment.

The behavior of the injected males raised apart from their fellows in reacting sexually toward humans, is very similar to that described by Craig (12) for male doves which matured in isolation, and by Lorenz (13) for hand-reared Great-Horned Owls, Ravens, "and other birds."

Females. The extensive work of Domm (14) on ovariotomy in the fowl seems to prove quite conclusively that the presence of a functional ovary is essential for the

normal sex behavior of the hen. Birds in which the left ovary and later the right compensatory gonad were removed assumed a neutral behavior resembling that of a capon. They exhibited none of the sexual behavior of either sex and were never observed to squat for a treading male. Allee and Collias (15) report the case of such a bird which, following injections with estradiol, squatted for a cock. This poulard was known to have previously repeatedly avoided the solicitations of sexually active males.

In the light of the above work it was thought possible to produce female sex behavior in estrogen-injected pullets, just as it was possible to induce masculinization in androgen-injected immature males. Our expectations were realized in *Experiment I* in which chicks injected with estradiol benzoate starting on the 15th day of age had all squatted for a treading male after 18 to 26 injections. In *Experiment II*, however, in which injections were started on the second day after hatching, no squatting was ever observed. The difference in behavior between the females of the two experiments presents a perplexing problem for which further study may provide a solution. It is possible, of course, that squatting occurred when the birds were not under observation. We do not consider this a likely occurrence. The male-female pairs were observed for longer periods of time than were those in *Experiment I*, and at various hours of the day, including early morning and late evening. It is felt that if squatting occurred at all it would have been seen in at least one case.

Body Weights

Males. Hamilton (3) reported that male chicks injected with testosterone propionate did not weigh as much as controls. These birds remained lighter in weight after the cessation of injections. Breneman (5), using smaller doses than were employed by Hamilton, found that body weight was essentially the same in control and injected chicks until after the end of treatment. The controls then became progressively heavier than the experimental birds. Our data confirm the results of Hamilton (3). The experimental males were significantly lighter than the controls on the day following the last injection. This difference in growth rate between the two groups was also apparent several weeks later.

Females. Pullets receiving placental hormone showed a greater final weight than the controls in the experiment of Juhn and Gustavson (16). Munro and Kosin (17) injected female chicks with 4 different estrogens and reported that there was no significant difference in weight between these birds and controls. Allee and Collias (15) injected estradiol in adult hens and obtained similar results. The data obtained in the present study showed that the 15-day-old chicks gained considerably more in weight than did the controls, by the end of the injection period. This difference in weight was less marked, however, 37 days after the cessation of injections. Chicks injected with heavy doses of estrogen starting at 2 days of age gained less weight than the controls by the end of treatment, while chicks of the same age given less hormone did not differ significantly from controls. It would appear, then, that the differences in dosage and age of the birds used would account for the conflicting results obtained by various workers.

Comb Growth

Males. Breneman (18, 19) found that after the injection of small doses of either testosterone propionate or dihydro-androsterone benzoate the chicks' combs did not retrogress, but continued to grow unabated. In the earlier work (18) he also reported that there was a temporary inhibition of testis growth which lasted through the injection period. In *Experiment II* we injected chicks with larger doses of testosterone

propionate than were used by Breneman, and over a considerably longer period of time. Within 3 weeks after the last injection the combs had retrogressed to a point where they were exceeded in size by the control combs by a considerable amount. This would seem to indicate that when large doses of androgen are injected into chicks, an inhibition of the testes is obtained which is effective for some time after injections are stopped.

Females. The findings of Dorfman and Greulich (20) and Munro and Kosin (17) indicate that none of the estrogens has an effect on comb growth of female chicks. Uotila (21), however, found that estrogen-injected pullets had combs slightly smaller than controls. Juhn, Gustavson, and Gallagher (22) reported that urinary estrogens did not affect significantly the combs of older birds. Allee and Collias (15) mention similar results obtained with estradiol, but add that heavy treatment with the estrogen caused comb involution. In our work we observed no difference between the combs of experimental and control female chicks.

SUMMARY

Male chicks injected with testosterone propionate exhibited all the sexual behavior patterns of the adult cock. Crowing appeared as early as the 4th day of age and treading was seen on the 15th day. The copulatory pattern was identical to that of the sexually mature bird. Injected males raised in isolation crowed and treaded as early as other treated birds. In addition, the experimenter could serve as 'a releaser' for some of the social and sexual reactions of these chicks which normally are directed only toward other birds of the same species.

Females injected with estradiol benzoate starting on the 15th day of age squatted for treading males after 18 to 26 daily treatments. The behavior was typically that of a sexually receptive hen. Chicks in which hormone treatment was initiated on the 2nd day of age did not exhibit this behavior.

The growth rate of experimental males was uniformly depressed. Augmentation or depression of growth rate was obtained in estrogen-injected females, depending upon the age of the birds, and the dosages administered.

The combs of experimental males retrogressed after the cessation of male hormone treatment to a considerably smaller size than those of controls. Female combs were unaffected by estrogen treatment.

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EFFECTS OF THE ADMINISTRATION OF ESTROGEN ON THE CONNECTIVE TISSUES OF THE GENITAL TRACT OF THE RAT[1]

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STUDIES CARRIED OUT in this laboratory have indicated that there are alterations in the relative and absolute amounts of reticulum and collagen in the genital tract of the female rat which may be associated with advancing age (1). In the uterus and cervix, and to a lesser degree in the vagina, there is primarily a transformation of reticulum into collagen and subsequently a further deposition of collagen, which is most rapid during the first three months but which continues throughout the life of the animal. These investigations have led to an inquiry into the nature of some of the factors which might be responsible for inducing these changes.

That the connective tissues of the reproductive organs are definitely affected by sex hormones has been indicated by a number of investigations. Korenchevsky and his collaborators (2, 3, 4), in their extensive studies of changes in the sex organs of rats after the administration of sex hormones, make frequent reference to fibrosis of the uterine mucosa associated with the increase in weight of the uterus itself. McEuen, Seyle and Collip (5) have also reported "enormous development of fibrous tissue in the submucosa" of the uterus of 6 castrated rats after the prolonged administration of estrogen. Suntzeff, Babcock and Loeb (6) have described as one of the effects of estrogenic administration in mice the deposition in the uterus and cervix of a hyaline substance having the staining properties of collagen, which is subsequently replaced by fibrillar connective tissue. In our own previous studies it has been observed that, in general, the uterus and cervix of virgin rats have a tendency to contain greater amounts of connective tissue than those of breeding rats (7). It was thought that this might be due to the effect of estrogen operating for a greater period of time in virgins, as judged by the criterion that the vaginae of virgin rats are cornified for a much greater proportion of the life span than are those of breeding rats (8).

Since these observations seemed to be of definite importance, we have studied the effects of estrogen administration on the uterus, cervix, and vagina of the rat by a method which differentiates reticulum from collagen. We were particularly interested in the action of estrogens on the transformation of reticulum into collagen which is known to occur in these organs.

MATERIAL AND METHODS

Immature female rats were used for these experiments. Some of these were castrates; the rest were not. The number of animals included in each series, the duration of the experimental periods, and the doses of the particular estrogenic substance used are given below.

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At necropsy blocks from the uteri and vaginae were fixed in both 10 per cent neutral formalin and Bouin's fluid, while the cervices were fixed only in formalin. Representative sections of the uteri and vaginae and serial sections of the cervices were cut. The formalin-fixed material was stained routinely by hematoxylin and eosin, by Goldner's modification of the Masson trichrome technique (9), and by Gömöri's modification of the Bielschowsky-Maresch silver impregnation method (10). The latter method differentiates reticulum, which is argyrophilic and appears black, from collagen, which is non-argyrophilic and stains various shades of rose red. The tissue fixed in Bouin's fluid was stained only by hematoxylin and eosin and by the trichrome method.

OBSERVATIONS

Injection of Estrogens into Immature Female Rats for a Short Period

Fourteen normal animals, 20 to 26 days of age, received daily subcutaneous injections of 200 R.U. of estradiol benzoate[2] for 15 days. Eleven littermate rats were

TABLE 1. AVERAGE WIDTH IN MILLIMETERS OF UTERI OF RATS INJECTED WITH ESTROGENS FOR 15 DAYS

Age at death (days)	Number of rats	Daily dose	Endometrium	Myometrium
		Normal rats, estradiol benzoate		
34–41	14	200 R.U.	0.71	0.72
34–41	11	Controls	0.50	0.25
40–41	3	10 R.U.	0.65	0.78
40–41	2	Controls	0.45	0.20
		Castrates, stilbestrol		
39	2	50 μg.[1]	0.69	0.64
35	2	100 μg.[1]	0.68	0.79
33–49	4	100 mg.[1]	0.60	0.71
33–39	6	Controls	0.29	0.15

[1] Total dose.

used as controls. In addition, 8 of 14 castrated females received variable amounts of stilbestrol,[3] as indicated in table 1. The remaining animals in the group served as controls. The effects of the estrogens on the genital tract are described below.

Uterus. Marked increase in size of the uterus was induced by the estrogens (table 1). Quantitatively the increase was strikingly greater in the myometrium than in the endometrium, but in all except 3 instances the endometrium also showed significant increases. It must be pointed out, however, that measurements do not necessarily reveal the actual amount of connective tissue present, for they do not give any indication of the density or looseness of the fibers constituting the stroma.

Representative sections of the uterus of an animal injected with estradiol benzoate and a normal control are shown in figures 1 and 2. The organs of the normal control females (fig. 1) showed the typical structure already described for animals of this age (1) and will be described here only briefly as a basis for comparison with the experimental animals. Immediately beneath the epithelial lining of the uterine cavity a reticular basement membrane and a subjacent zone (subepithelial zone), made up of a delicate reticular meshwork, usually comprised about one-fourth to one-third of the width of the endometrium. Abundant stromal cells were present in the interstices of

[2] The estradiol benzoate was furnished by the Schering Corporation through the courtesy of Dr. Erwin Schwenk, Bloomfield, New Jersey.

[3] The stilbestrol was kindly supplied by Dr. J. A. Morrell of E. R. Squibb and Company of New Brunswick, New Jersey.

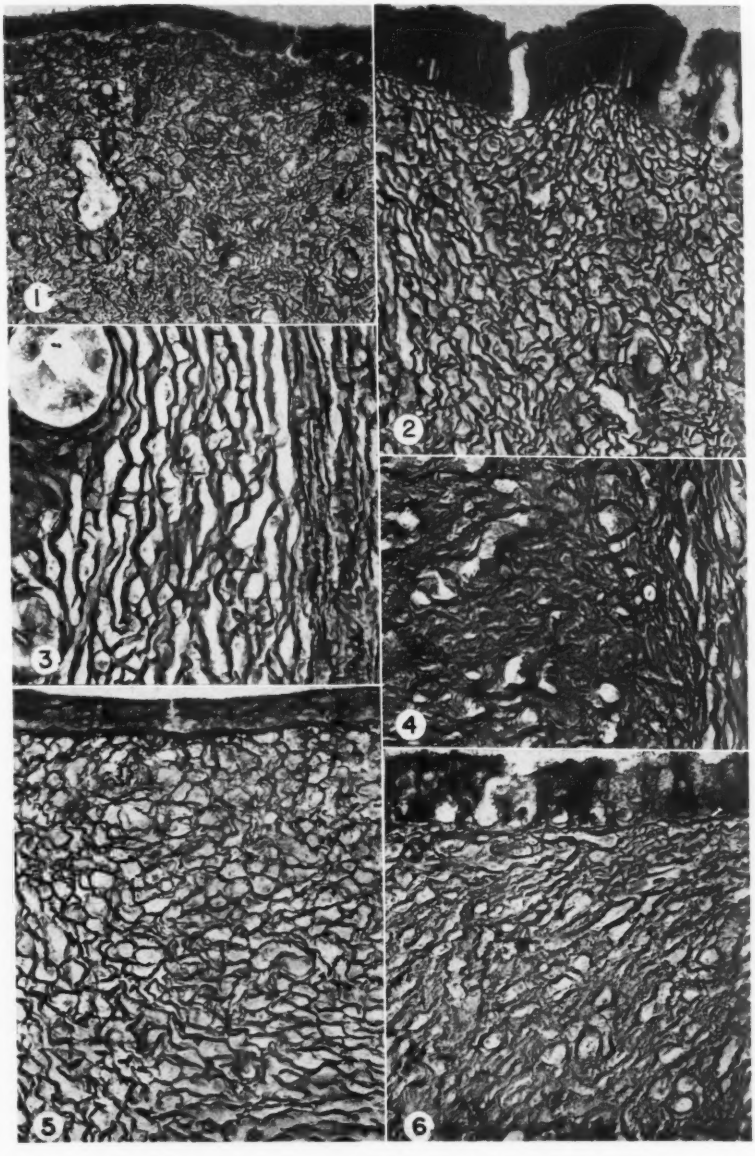

Fig. 1. Uterus of a 35-day-old normal rat. Note reticular basement membrane under epithelium and around gland, reticular network in subepithelial zone, and fine collagenous meshwork (gray) deep in endometrium. Gömöri stain. ×350. Fig. 2. Uterus of an injected animal of comparable age but which received daily 200 r.u. estradiol benzoate for 15 days. Note meshwork of coarse fibers. The only reticulum present was a very narrow subepithelial network. The edges of the collagenous fibers are apparently denser than the rest of the fiber and photograph black instead of gray, giving the erroneous impression that reticulum is present throughout the endometrium. Gömöri stain. ×350.

Fig. 3. Uterus of a normal animal 179 days old. Observe the definite fibers running parallel to

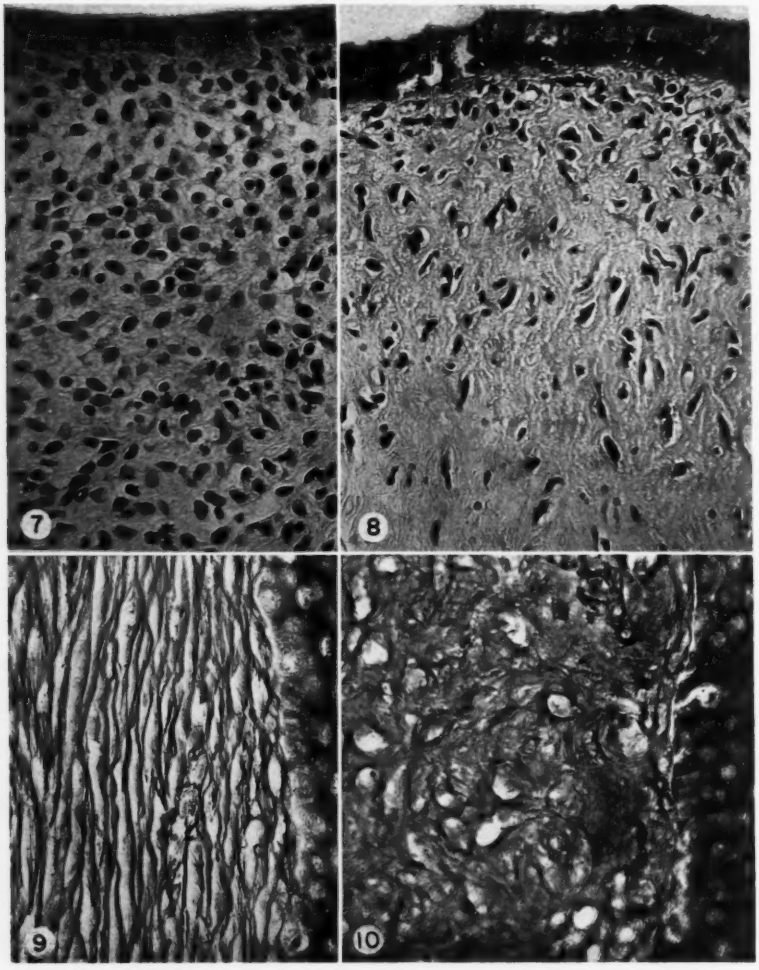

Fig. 7. SECTION OF SAME UTERUS AS IN FIGURE 3 stained by Masson trichrome method to show cells in superficial endometrium of normal 6-month-old rat. ×350. Fig. 8. SAME UTERUS AS IN FIGURE 4 stained by Masson trichrome method. Observe shrunken, distorted nuclei of fibroblasts and relative acellularity. Several elongated cells in field are wandering cells. ×350.

Fig. 9. SECTION OF CERVICAL CANAL OF NORMAL RAT 330 DAYS OLD. Note collagenous fibers. Gömöri stain. ×350. Fig. 10. SECTION OF CERVICAL CANAL OF RAT OF SAME AGE but injected daily with 200 R.U of estradiol benzoate for 309 days. Observe dense, sheet-like collagen. Gömöri stain. ×350.

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adjacent muscular coat from which endometrium is clearly demarcated. Gömöri stain. ×350. Fig. 4. UTERUS OF A RAT OF THE SAME AGE BUT INJECTED DAILY WITH 200 R.U. ESTRADIOL BENZOATE FOR 158 DAYS. Observe dense collagen and irregular border between endometrium and myometrium. Gömöri stain. ×350.

Fig. 5. SAME UTERUS AS IN FIGURE 3 SHOWING PRESENCE OF A WELL DEFINED RETICULAR BASEMENT MEMBRANE AND AN UNDERLYING MESHWORK OF RETICULUM. The edges of collagenous fibers in deeper portion have photographed black presumably due to greater density and should not be mistaken for reticulum. Gömöri stain. ×350. Fig. 6. SAME UTERUS AS IN FIGURE 4 REVEALING THAT ONLY AN INCOMPLETE RETICULAR BASEMENT MEMBRANE IS PRESENT and that collagen is dense even in the superficial portion of endometrium. Gömöri stain. ×350.

the meshwork. Delicate collagenous fibers were intermixed with the argyrophilic fibers; it has been reported previously that these formed as the result of transformation of reticular fibrils. Deeper in the endometrium a fine collagenous meshwork predominated, although reticular fibrils were present about the glands and the blood vessels. Although the stroma was cellular throughout the entire width of the endometrium, it was definitely more so in the subepithelial, reticular zone than in the region adjoining the myometrium.

In the injected rats it was evident that estrogen had induced a hastening of the transformation of endometrial reticulum into collagen, and that there was an intensification of the deposition of the latter with the formation of coarser, denser fibers (fig. 2). In most instances the fairly wide subepithelial zone of reticulum which was present characteristically in the controls had been largely replaced by collagen. The reticular basement membrane still persisted, and in some rats there remained a narrow zone of underlying reticulum, which, however, almost immediately blended with a well defined collagenous meshwork that was much thicker and composed of coarser fibers than that found in the controls. The collagenous fibers were often disposed in a radial fashion, whereas normally the fibers adjacent to the inner muscular coat run a more or less circular course. The endometrium was cellular throughout, but the cells, ordinary spindle-shaped fibroblasts, tended to be arranged with no obvious regional distribution as in the control uteri. This was correlated both with the deficiency of subepithelial reticulum, in the delicate network of which there are characteristically abundant large, round stromal cells, and with the lack of any appreciable difference in the thickness of the collagenous fibers that composed both the superficial and deep regions of the endometrium in the estrogen-treated rats. We have repeatedly observed that the degree of cellularity of the endometrial connective tissue is related to the thickness of the fibers composing the meshwork, namely, the thicker and more compact the fibers, the fewer the cells present; the thinner and more loosely interwoven, the more abundant are the cells enclosed in the fibrous mesh. In the normal endometrium, the thickest and most closely packed fibers are in the deepest portion; this is also the least cellular.

Associated with the striking growth of the muscular coats, certain changes were noted in the connective tissue fibrils about the muscle cells. In the inner (circular) muscular layer the reticular fibrils around the muscle cells were in a number of instances replaced by delicate rose-colored fibers indicating transformation of reticulum into collagen. There also appeared to be a definite increase in the number and width of the collagenous fibers surrounding the blood vessels which lie between the two coats of muscle. In the outer (longitudinal) layer the fibrils surrounding the muscle cells were in most instances argyrophilic and were thus, as in the controls, purely reticular in nature.

In 3 animals injected for 15 days with daily doses of 10 R.U. of estradiol benzoate instead of 200 R.U., the uterine stromal changes approximated both qualitatively and quantitatively those observed with the much larger dose (table 1). These findings suggest the need of further studies to determine the threshold of sensitivity of the uterine connective tissues to estrogenic stimulation.

The administration of stilbestrol to castrated females produced changes which were in general similar to those already described for the estradiol benzoate. The difference in degree could be accounted for by the variation in dosage of the estrogens. The amount of stilbestrol which most nearly produced the results noted for the estradiol was, in these particular animals at least, 100 micrograms as the total dose.

Cervix. The structure of the cervices of the control rats which were used for determining the differences resulting from estrogenic administration resembled closely that of normal rats of this age period previously described (1). Briefly, the stroma of

the uterine portion of the cervix resembled that of the uterus itself and contained an appreciable amount of reticulum beneath the lining epithelium. This tissue gave way to purely collagenous fibers in the deeper portion bordering on the circular muscle layer. Sometimes the uterine type of stroma continued for a short distance into the vertical cervix (region of the cervical canals) but sooner or later, abruptly or gradually, the connective tissue under the stratified epithelium of the cervical canals, except for the occasional persistence of a basement membrane, became changed into deep red, coarse fibers running parallel to the canal and finally in diversified directions in the regions of the lips and fornices. The stroma was generally cellular, more so in the regions where reticulum was found than in those where collagen predominated. The reticular meshwork of the cervical muscular coats was prominent, particularly so in the uterine cervix and in the upper part of the vertical portion. Caudally, especially in the region of the lips, there was a general diminution of reticulum associated with a decrease in the number of muscle cells, which were isolated into small units by coarse collagenous fibers. There continued, however, to be an appreciable amount of muscle in this region.

The changes in the cervices of the estrogen-treated animals paralleled those found in the uterus, namely, a reduction in the amount of subepithelial reticulum, an accelerated deposition of collagen, and a growth of the cervical musculature. The uterine portion retained variable but usually slight amounts of reticulum. In the majority of cases the argyrophilic basement membrane represented the only reticulum, with the exception of the basket-like meshwork about the muscle cells. Sometimes even the basement membrane was doubtfully argyrophilic. The density and the pattern of the collagenous fibers approximated that found in the corresponding uterus.

In the vertical portion the collagenous fibers became coarser, more compact, and were irregularly arranged due to the bizarre downgrowths of the highly stimulated lining epithelium. Progressively downward from the uterine end the muscle reticulum was gradually replaced by narrow collagenous fibers, and these tended to separate the muscle cells into many small aggregates; this was particularly true in the region of the lips. Where the muscle coat continued into the vagina proper, the fibrils about the muscle cells usually became again intensely argyrophilic. The reason for this interesting finding is not known. The entire stroma was abundantly cellular.

Vagina. In contrast to the well known, striking changes in the epithelium produced by estrogen, the alterations in the connective tissues were less obvious and were of a quantitative rather than a qualitative nature. This was to be expected, as the previous observations of normal animals (1) had indicated that the vagina of even a 5-day-old normal rat was definitely collagenous. In many instances, in the injected as well as in the control animals, an argyrophilic basement membrane of variable degree of continuity underlay the epithelium, but in general the only reticulum present in the vagina was that surrounding the muscle cells, which appeared to be quite conspicuous. The stroma was very cellular. In the Bouin-fixed tissue the collagen tended to be arranged in a continuous sheet of fine fibrils closely packed together, whereas in the formalin-treated material broad and separated fibers were found. This difference has already been commented upon and an explanation suggested (1).

Prolonged Administration of Estrogen

Seven normal animals survived daily injections of 200 R.U. of estradiol benzoate[2] for 57, 117, 158, 309 and 320 days. Littermate controls were autopsied at the same periods. In spite of the wide variation in the length of the injection periods, the effects upon the genital tract were essentially similar.

Uterus. Histological comparison of the organs of the estrogen-injected animals

with those of the littermate controls showed clearly that significant alterations in the connective tissues of the uterus had occurred with the prolonged, uninterrupted administration of estrogen. Whereas the collagenous tissue in the endometrium of all the controls was arranged in the characteristic architectural pattern of fibers surrounding the glands in whorl-like fashion and running circularly in the region adjacent to the muscular coat (fig. 3), the endometrial collagen of the estrogen-treated animals was dense and hyaline-like (fig. 4). The silver preparations revealed that the collagen was not truly amorphous but rather made up of a sheet of very fine fibrils closely packed together (fig. 4 and 6). In contrast to the constant presence of a subepithelial network of reticulum of variable width in the control uteri (fig. 5), practically no reticulum was found in the subepithelial zone of the experimental organs (fig. 6), and not infrequently even the basement membrane appeared collagenous rather than reticular. There was a pronounced difference in the appearance of the stromal cells of the experimental and control uteri. In striking contrast to the abundant cells of normal appearance usually found in the endometrium (fig. 7), the stromal cells in the endometrium of the estrogen-treated animals were shrunken, their nuclei elongated and bizarre-shaped (fig. 8). Whereas the control uteri contained numerous nests of glands, the organs of the injected females showed negligible development of glands; the relative absence of glands gave the endometrium a markedly uniform appearance.

The myometrium as a whole was demonstrably thicker in the injected animals than in the normal controls, but in the latter the inner coat was much more clearly demarcated from the adjacent endometrium (fig. 3). This was due to the fact that in the experimental organs there was a noticeable deficiency of reticulum in the inner or circular myometrial coat, and the connective tissue about the muscle cells was largely composed of narrow collagenous fibers which grew in partly from the adjacent endometrium and thus produced an irregular boundary between the two tissues (fig. 4). In the longitudinal coat as well, unlike that in rats injected with estrogen for the 15-day period, reticulum was also greatly reduced in amount and replaced by thin collagenous fibers; in contrast, the connective tissue framework of the longitudinal myometrium of the control organs was predominantly argyrophilic even in the oldest animal. It appears significant that uteri such as were found in the estrogen-treated animals had been observed only among females 18 months or older (1).

Cervix. In general the alterations in the connective tissues of the cervices of the injected animals were similar in nature to those found in the uterus. Changes were evident even in the animal injected for only 2 months but were more marked in the animals surviving the longer periods. Grossly the cervices were demonstrably larger and were strikingly more firm than those of the littermate controls with which each was compared.

Histologically in the silver preparations no reticulum was present in the uterine portion of the cervix of the estrogen-treated animals, whereas even in the 330-day-control the subepithelial zone was reticular. The stroma appeared as a dense sheet of fine collagenous fibrils so closely packed together that it appeared homogeneous, especially in those rats which had been injected for the longer periods. In contrast, the cervical stroma in the controls, although fairly dense, was made up of thick collagenous fibers similar to those in the uterus. After injection with estrogen for more than 300 days, the coarse fibers, which are normally arranged parallel to the overlying stratified epithelium of the cervical canals, were in great part replaced by a dense, sheet-like stroma (compare fig. 9 with fig. 10).

The stromal cells were likewise significantly affected. In the control animals the stroma remained quite cellular, while in the injected rats it was relatively acellular, and most of the cells which were present were shrunken.

Although the reticulum of the cervical musculature was fairly well preserved in those animals injected for 2 to 4 months, it was to a large extent replaced by thin collagenous fibers in the animals injected for longer periods. Associated with this disappearance of muscle reticulum, there were atrophy and vacuolization of the muscle cells. There was such an extensive overgrowth of dense collagenous tissue that the individual muscle bundles were widely isolated from each other, and in the lower part of the cervix (the vaginal portion) the muscle was practically entirely crowded out by collagenous tissue. In the female treated for 309 days, the longitudinal muscle layers which are usually seen at either side of the cervical canals just under the subepithelial connective tissue were almost entirely replaced by dense, sheet-like fibrillar collagen identical with that in the uterus and uterine cervix. The deficiency in muscle was in distinct contrast to the prominence of the musculature in the cervices of the controls; associated with the abundance of normal muscle cells, the embracing network of connective tissue was clearly reticular in the upper region, and was observed to be in part collagenous only in the lower, vaginal portion of the cervix, as has been observed in many normal animals of this age range (1).

It may be mentioned that the pathological effects of estrogen on the upper genital tract in producing epithelial desquamation and inflammation, which has been so fully described elsewhere (11) were quite apparent, and evidence of a low-grade chronic inflammatory reaction was present in these animals. It may also be added that the squamous metaplasia of the uterine epithelium which has been reported by Zondek (11) and McEuen (12) to occur after long-continued administration of estrogen was observed in 3 rats of this group.

Vagina. Except for an apparent hastening in the transformation of the reticulum surrounding the individual muscle cells of the vaginal musculature into collagenous fibrils, and for a thickening of the vaginal wall, no important changes were found in the connective tissues of this organ. The pattern in which collagen was deposited was not demonstrably different in the estrogen-treated animals from the controls and was similar to that which occurred in rats injected for shorter periods.

DISCUSSION

It appears to be clear that at least one of the causal factors involved in the transformation of reticulum into collagen and continued deposition of collagenous tissue in the genital tract is the estrogenic hormone. That this is not the only sex hormone which may be concerned in these processes has been indicated by the experiments of Korenchevsky and others (2, 3, 4) as well as by preliminary work in this laboratory, in which it was found that the administration of testosterone propionate to immature rats also hastened the deposition of endometrial collagen. These findings would suggest that in the normal female rat the amount, character and relative proportion of the connective tissues in the reproductive tract is regulated at least by endogenous estrogen and perhaps also by certain androgens. The rôle which sex hormones other than estrogen may play is not definitely ascertained, but since progesterone is known to modify the effect of estrogen on the vagina and uterus, it would seem that this hormone may at least exert an indirect effect.

The transformation of reticulum into collagen observed with estrogen administration is associated with concomitant change in the structure of the stromal cell within the fibrillar network, from a rounded cell with a large, highly vesicular nucleus to the spindle-shaped form of the ordinary fibroblast. This difference in appearance is thought to indicate an associated functional change in the cell which must be related in some way to the type of fibers with which the cells are related. The whole relationship between the nature of the stromal cells and the type of the intercellular fibers merits further investigation.

Finally, the observations on the effect of prolonged administration of estrogenic hormone to rats have indicated that uninterrupted stimulation by this hormone for an extended period of time may lead to a hastening of the aging processes in the genital tract. If atrophy of cells and increase in amount and density of collagen in organs can be considered as criteria of aging, then the uterus and cervix of the estrogen-treated rats had aged considerably beyond their normal period of life. Mention has already been made of the fact that in the study of a large series of normal animals, uteri similar in structure to those of rats receiving extended injections of the estrogenic hormone were observed only among animals over 18 months old. To what extent the constancy of the stimulation was responsible for these changes is as yet undetermined. Possible modifications by periodic interruption of estrogenic treatment or by alternation with progesterone must await further study.

SUMMARY AND CONCLUSIONS

Administration of large doses of estradiol benzoate to normal, immature rats for 15 days induced a hastening of the transformation of reticulum into collagen and an intensification in the deposition of collagenous tissue in the uterus and cervix, with the formation of coarser, denser fibers. The connective tissue of the vagina under such conditions differed from that of the control organ only in amount rather than in the character of the fibrous pattern. The administration of stilbestrol in variable doses to young castrated rats for approximately two weeks immediately after operation produced changes in the reproductive tract similar to those produced by the injection of estradiol benzoate into unoperated animals.

Prolonged, uninterrupted administration of estrogenic hormone to normal rats further resulted in a change in the architectural pattern of collagenous tissue from the characteristic one of a meshwork of well defined fibers to a dense, almost homogeneous fibrillar sheet. Associated with this was a prominent shrinkage of the cellular components. In general, it may be said that long-continued treatment with estrogen appeared to hasten the aging processes in the genital tract.

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OCCURRENCE OF URINARY CALCULI IN INBRED STRAIN (C_3H) OF MICE TREATED WITH ESTROGEN[1]

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INVESTIGATORS WHO HAVE TREATED castrated and noncastrated male mice with estrogen have observed various pathologic changes in the genito-urinary tract, including *a*), slight increases in the dried weight of the kidneys, independent of possible inflammatory changes (1); *b*) hydronephrosis and hydroureter, sometimes complicated by infection and suppuration which led, in turn, to pyonephrosis and pyoureter (2, 3); *c*), dilatation of the urinary bladder (3, 4, 5), urinary retention, sometimes associated with purulent cystitis, and squamous metaplasia of the bladder epithelium with downgrowths extending to the muscular coat (2); *d*), squamous metaplasia of the epithelium of the prostatic urethra and periurethral glands (6); *e*) squamous metaplasia of the epithelium lining the prostate glands (4–11), seminal vesicles (5, 9, 10, 11), coagulating glands (6, 10, 11), and bulbo-urethral glands (12), sometimes with keratinization of the epithelium and suppuration in the glandular lumina; *f*) increase in the fibrous tissue stroma of the prostate glands (6), and thickening of the submucosa of intraprostatic part of the vas deferens (5); g) extrusion of the penis (5); *h*), proliferation of the interstitial cells of the testes, sometimes with the subsequent development of benign and malignant tumors arising from these cells (13, 14); i) proliferation of Müllerian duct remnants into newly-formed structures, with squamous metaplasia and keratinization of the epithelium (15).

The type of pathologic change found by the various investigators differed according to the strain of the mice used, the type of estrogen administered, the strength and duration of the dose, and whether or not the animals had been castrated. Many of the changes produced were reversible and the affected organs returned to normal after treatment was discontinued (9, 10).

In a series of male C_3H mice treated with estrogen we observed many of the changes in the genito-urinary tract reported by other investigators. We also observed a change which, so far as we know, has not previously been reported, namely, a high incidence of urinary tract calculi. This accidental observation, made in the course of an experimental study of estrogen-induced mammary gland cancer, was the subject of a previous preliminary report (16). We are now making a further report of our findings, and are also recording some of the associated pathologic-anatomic changes observed in the genito-urinary tract.

MATERIALS AND METHODS

The studies reported in this paper were carried out on 365 strain C_3H mice, all of which were necropsied. The parent mice were obtained in 1938 from the Jackson Memorial Laboratory at Bar Harbor, Maine, and propagation in New Orleans has been carried on only by brother to sister matings. The animals were fed regularly with fresh Purina dog chow, and occasionally with lettuce. Water was always available through drinking tubes.

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One hundred ninety-nine nonbreeding mice, ranging in age from 4.1 to 21.2 months, were used as experimental animals, and 166 breeding and nonbreeding animals ranging in age from 2.4 to 19.8 months were used as controls. The experimental animals were injected subcutaneously, beginning at the age of 10 to 14 days, with crystalline α-estradiol benzoate in sesame oil[2] and were divided into 7 groups, as follows.

Group 1; 21 male animals which were given 2 doses each of 1500 R.U. of α-estradiol benzoate on alternate days, a total of 3000 R.U. (0.498 mg.). *Group 2;* 18 males and 3 females which were given 100 R.U. (0.0165 mg.) at weekly intervals for 4 weeks. *Group 3;* 30 males and 3 females which were given similar injections for 8 weeks. *Group 4;* 19 males and 5 females which were given similar injections for 12 weeks. *Group 5;* 27 males and 3 females which were given similar injections for 16 weeks. *Group 6;* 25 males and 5 females which were given similar injections for 20 weeks. *Group 7;* 29 males and 11 females which were given weekly injections of 100 R.U. continuously throughout their lifetime. The periods of treatment ranged from 17 to 62 weeks and the average amount administered was 3259 R.U. (0.541 mg.).

Although treatment was terminated in *groups* 1 to 6 inclusive at the end of the stated periods, the animals lived thereafter for an average of 14.5 months in *group* 1, 12.2 months in *group* 2, 7.9 months in *group* 3, 8.9 months in *group* 4, 5.0 months in *group* 5, and 4.8 months in *group* 6.

The 166 control animals consisted of 26 nonbreeding and 47 breeding males, and 25 nonbreeding and 68 breeding females.

Although some deaths occurred spontaneously, most of the animals were killed with chloroform when they appeared seriously ill. Representative tissues from each animal were fixed either in Helly's fluid or in 10% formalin. Microscopic studies of organs of the genito-urinary tract were made on tissues embedded in paraffin and stained with hematoxylin and eosin. To date examinations have been completed on 43 animals, the number including representatives of each of the various experimental and control groups.

The presence of calculi was determined by gross examination of the urinary tract. The calculi were fixed in formalin with the tissues in which they were contained, and then were removed and air-dried at 100°C. until complete desiccation occurred. The dry calculi were weighed, crushed, and dissolved in dilute hydrochloric acid, after which the insoluble material was filtered off and examined microscopically. In every case all of the calculous material found in the urinary tract of each animal was used for chemical analysis. The qualitative analyses were carried out by the method of Domanski (17). For the quantitative analyses the calcium was precipitated from an aliquot as calcium oxalate and determined quantitatively by titration with standard potassium permanganate. The magnesium was precipitated as the oxalate in 85% acetic acid and determined by titration with standard $KMnO_4$. The phosphorus was determined by the routine clinical method of Youngburg and Youngburg (18) for blood phosphate.

To date qualitative chemical analyses have been made on calculi from 22 treated males and 1 control female, and quantitative chemical studies have been made on calculi from 14 of the same treated male animals.

RESULTS

Urinary Calculi

Incidence. Urinary calculi were found in 4.1% of the male and 5.4% of the female control animals. They were observed in 4.0% of the 25 nonbreeding females, in 5.9%

[2] Alpha-estradiol benzoate (Progynon-B) was supplied by the Schering Corporation, Bloomfield, N. J.

TABLE 1. URINARY TRACT CALCULI IN UNTREATED CONTROL C3H MICE

		Males			Females		
		Non-breeding	Breeding	Total	Non-breeding	Breeding	Total
Age at death, months	Calculi	2.4–8.9 Av. 4.9	—	2.4–8.9 Av. 4.9	12.7	11.3–14.7 Av. 13.0	11.3–14.7 Av. 12.8
	No calculi	4.0–16.1 Av. 11.4	6.0–18.7 Av. 12.5	4.0–16.1 Av. 12.2	6.3–19.8 Av. 10.7	4.8–18.9 Av. 11.6	4.8–19.8 Av. 11.4
Number of animals	Calculi	3	0	3	1	4	5
	No calculi	23	47	70	24	64	88
	Total	26	47	73	25	68	93
Per cent with calculi		11.5	0	4.1	4.0	5.9	5.4

of the 68 breeding females, and in 11.5% of the 26 nonbreeding males. No calculi were observed in the 47 breeding male controls (table 1).

The incidence of calculi in the treated male mice was 43.7% (74 of 169 animals) and in the females it was 3.4% (1 of 30 animals). The incidence in the male animals varied according to the dose of estrogen and the mode of its administration. The animals in *groups* 1, 2 and 7 showed an almost equal incidence of calculi, 33.3, 27.7 and 27.5% respectively. The animals in *groups* 3, 4, 5 and 6 showed a higher incidence, 43.3, 52.6, 59.2, and 60.0% respectively. The single treated female animal which developed calculi was given 1800 R.U. (0.297 mg.) of estrogen in weekly doses throughout its lifetime of 4.83 months (table 2).

TABLE 2. INCIDENCE OF URINARY TRACT CALCULI IN STRAIN C3H MICE TREATED WITH ESTROGEN

Group	Total dose, R.U. and mg.	Occurrence of Calculi							
		Males				Females			
		No. with	No. without	Total	% with	No. with	No. without	Total	% with
1	3000 R.U. 0.498 mg.	7	14	21	33.3	0	0	0	0
2	400 R.U. 0.066 mg.	5	13	18	27.7	0	3	3	0
3	800 R.U. 0.132 mg.	13	17	30	43.3	0	3	3	0
4	1200 R.U. 0.198 mg.	10	9	19	52.6	0	5	5	0
5	1600 R.U. 0.265 mg.	16	11	27	59.2	0	3	3	0
6	2000 R.U. 0.333 mg.	15	10	25	60.0	0	5	5	0
7	1700 to 6200; av. 3259 R.U. 0.541 mg.	8	21	29	27.5	1	10	11	9.1
Total		74	95	169	43.7	1	29	30	3.4

Age. The average age at death of all control males which developed calculi was 4.9 months; the youngest animal in this group was a nonbreeding mouse 2.4 months old and the oldest was a nonbreeding mouse 8.9 months old. The average age of all control females which developed calculi was 12.8 months. The youngest animal in this group was a breeding mouse 11.3 months old and the oldest was a breeding mouse 14.7 months old. Detailed infromation as to the age of the control mice is shown in table 1.

The average age at death of all treated males which developed calculi was 9.5 months, the age range being from 4.1 to 19.8 months. The single treated female mouse which developed calculi was 4.8 months old. With the exception of one group (*group* 6), in which the total dosage of estrogen amounted to 2000 R.U. (0.333 mg.), the average age at death of the animals which developed calculi in each group was below the average age of the animals which did not develop them. Detailed information as to the age of the treated animals is shown in table 3.

TABLE 3. AGE OF STRAIN C_3H MICE TREATED WITH ESTROGEN

Group	Total dose in R.U. and mg.	Age, at which injections ended, mo.	Age in Months at Death			
			Males		Females	
			With calculi	Without calculi	With calculi	Without calculi
1	3000 R.U. 0.498 mg.	0.53	6.3–19.8 Av. 13.5	10.7–21.2 Av. 15.7		
2	400 R.U. 0.066 mg.	1.06	8.9–17.1 Av. 11.8	8.9–18.8 Av. 13.8		12.8–13.8 Av. 13.2
3	800 R.U. 0.132 mg.	2.10	4.4–19.5 Av. 8.8	5.1–21.2 Av. 11.04		14.1–15.1 Av. 14.5
4	1200 R.U. 0.198 mg.	3.00	8.2–15.9 Av. 10.6	7.1–19.4 Av. 13.3		6.4–12.8 Av. 9.2
5	1600 R.U. 0.265 mg.	4.04	4.1–13.4 Av. 7.9	6.0–17.8 Av. 10.7		7.2– 9.8 Av. 8.0
6	2000 R.U. 0.333 mg.	4.94	5.4–15.5 Av. 9.8	5.4–14.1 Av. 9.7		5.5–10.0 Av. 8.1
7	1700 to 6200, av. 3259 R.U. 0.541 mg.	8.19	4.2–12.5 Av. 7.5	4.3–14.6 Av. 8.4	4.8	4.1–11.3 Av. 7.4
Totals			4.1–19.8 Av. 9.5	4.3–21.2 Av. 11.6	4.8	4.1–15.1 Av. 9.2

Location and character. In the control mice calculi were usually found only in the bladder (fig. 1); occasionally they were found in the urethra as well. In the treated mice they were most commonly found in these locations (fig. 2) but were also present in the pelves of the kidneys and in the ureters. In some animals calculi were found in only one of these sites, in others they were present in two or more locations (fig. 3). Speaking generally, the greatest number and the widest distribution of the concretions in the urinary tract was observed in the groups in which the highest incidence of calculi was observed.

The individual calculi in the treated animals varied in size from tiny, gravel-like fragments to concretions 0.5 cm. in diameter. They were rough, white, moderately

soft, chalky, and sometimes laminated. Often several calculi, cemented together with a soft, chalky material or a tenacious mucoid substance, formed a cast of the entire bladder (fig. 3). Usually from 3 to 10 calculi were present in the bladder. Innumerable fine particles were sometimes observed in the bladder and in the pelves of the kidneys.

The individual calculi in the control animals were uniformly 0.1 to 0.3 cm. in diameter. In contrast to those found in the treated animals, they were smooth, hard, oval and discrete (fig. 1). They were white in color. In general, the number of calculi

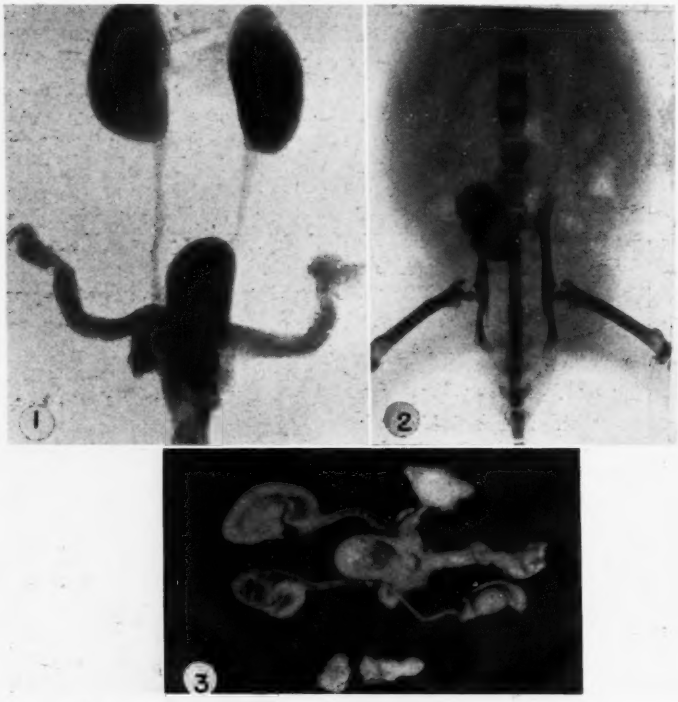

Fig. 1. ROENTGENOGRAM OF GENITO-URINARY TRACT OF CONTROL BREEDING FEMALE 14.6 MONTHS OLD. Four, smooth, oval discrete calculi in urinary bladder. Fig. 2. ROENTGENOGRAM OF TREATED LIVING MALE 8.7 MONTHS OLD (*group 6*). Numerous urinary calculi in bladder. Fig. 3. GENITO-URINARY TRACT OF MOUSE IN FIGURE 2. Urinary calculi in left renal pelvis, left ureter, bladder and urethra. Removed calculi form cast of bladder and urethra. Note hydronephrosis, hydroureter and thickened bladder wall. Left orchitis present.

in the control animals was less than the number in the treated animals, 4 being the largest number found in any single control animal.

The calculi in both treated and control animals were of the 'bone earth' type. The inorganic material consisted of calcium and magnesium phosphates. The calcium component varied from 2.4 to 25.3%, the magnesium component from a trace to 10.4%, and the phosphate component from 28.6 to 53.3%. The calcium present in all stones averaged 11.9%, the magnesium 6.5%, and the phosphates 35.3%. No other inorganic constitutents were found in appreciable amounts.

The material which was insoluble in HCl was found on chemical analysis to consist of organic material and on microscopic examination to be composed of cellular and amorphous debris. The material, together with the water of crystallization, was pres-

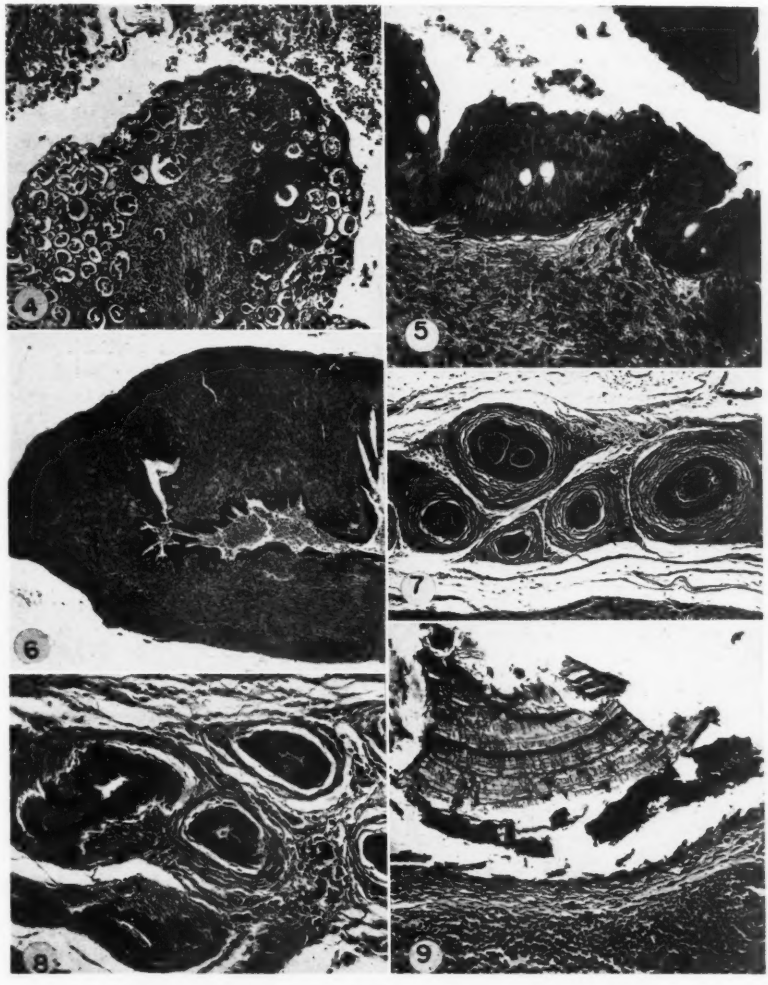

Fig. 4. TIP OF RENAL PYRAMID OF TREATED MALE (*group* 3). Necrosis and dark staining colonies of bacilli in kidney tissue. Inflammatory exudate in pelvis. ×108. Fig. 5. BLADDER MUCOSA OF TREATED MALE (*group* 5). Squamous metaplasia, epithelial downgrowths and pseudo-acinous formation in epithelium. Fibrosis and inflammatory cells in mucosal stroma. ×172. Fig. 6. SEMINAL VESICLE OF TREATED MALE (*group* 7). Atrophy of the organ and fibrosis of wall. ×81. Fig. 7. COAGULATING GLAND OF TREATED MALE (*group* 7). Squamous metaplasia of epithelium and periacinar fibrosis. ×90. Fig. 8. PROSTATE GLAND OF TREATED MALE (*group* 7). Stratification of columnar epithelium and periacinar fibrosis. ×108. Fig. 9. BLADDER OF TREATED MALE (*group* 6). Microscopic calcium depositions on fibrin in lumen. ×162.

ent in percentages varying from 13.9 to 64.4 the average proportion for all the calculi examined being 46.2%.

Histopathologic Changes in the Tissues of the Genito-urinary Tract

Kidney. In occasional instances the kidneys of the treated animals which had developed calculi were normal. A few showed mild to marked hydronephrosis, but the majority showed chronic pyelonephritis, with or without acute pyelitis and foci of

suppuration in the pyramids. Colonies of bacilli were often present in the pyramids in which necrosis was observed (fig. 4). The pelvic epithelium showed stratification with from 2 to 8 layers of cuboidal or polygonal cells; squamous metaplasia with keratinization was also occasionally noted. The pelves often contained exudates consisting of varying proportions of inflammatory cells, desquamated epithelial cells, necrotic cellular debris and of threads of fibrin, upon which deposits of calcium were occasionally noted.

The kidneys of treated animals which did not develop calculi were usually either normal or showed cortical scars which were probably the result of pyelonephritis. The scars were more prominent in the older animals. Acute pyelitis and acute pyelonephritis were observed in some instances.

Urinary bladder. In most instances the bladders of animals which had developed calculi showed an absence of the normal mucosal folds. In some instances the folds were blunt and thick. Squamous metaplasia of the epithelium, as well as epithelial downgrowths and pseudoacinous formation, were observed almost uniformly. The mucosal stroma was usually increased in amount and infiltrated with varying proportions of lymphocytes, plasma cells, macrophages and polymorphonuclear leukocytes (fig. 5). Foci of lymphocytes, plasma cells and macrophages were commonly present in the muscle coat, and areas of replacement fibrosis were occasionally noted. Ulcerations with acute inflammation were common.

The urinary bladders of treated animals without calculi were usually normal, although fibrosis of the stroma of the mucosa and slight downgrowths of the bladder epithelium were occasionally observed.

Seminal vesicles. The seminal vesicles of the older breeding males in the control group showed slight reductions in size, together with increased thickness and hyalinization of the fibromuscular wall. Variable reductions in size were also noted in the seminal vesicles of the treated animals. In general, the animals which lived the longest after treatment was discontinued showed the least reduction in the size of the seminal vesicles; animals which were treated until death showed marked atrophy of these organs, as evidenced by great reduction in the size of the lumen, as well as fibrosis and hyalinization of the thickened wall (fig. 6).

Squamous metaplasia of the epithelium of the seminal vesicles was not observed in either group. Inflammation was occasionally encountered in the treated mice but was not observed in the control animals.

Coagulating glands. The coagulating glands of the treated animals often showed stratification of the columnar epithelium, associated in many instances with the formation of new acini which partially filled the lumen. Animals which received treatment until death showed squamous metaplasia of the epithelium and periacinar fibrosis, whether or not urinary tract calculi and inflammation were present (fig. 7). When calculi and inflammatory changes were present in the urinary tract, similar metaplasia was often observed in animals which had received either 1600 or 2000 R.U. of estrogen. Suppuration within the acini and in the supporting tissue of the coagulating glands was observed in some of the treated animals in all groups. Marked infiltrations of lymphocytes, plasma cells, macrophages and foam cells, as well as fibrosis, were commonly present in the supporting tissues, especially when urinary tract calculi and infection were present. Cholesterol clefts were occasionally present in these tissues. None of these changes was observed in the coagulating glands of the control animals.

Prostate glands. The prostate glands of the animals which received estrogen treatment until death showed stratification of the columnar epithelium and an increase in periacinar stroma (fig. 8), although the latter change was less marked in the prostate than in the coagulating glands. Infiltration of the stroma with lymphocytes, plasma

cells and macrophages was also noted. All of these changes tended to persist even after treatment was discontinued if urinary tract calculi and infection were also present. When urinary tract calculi and infection were absent, the prostate glands usually appeared normal if treatment had been terminated for some time. None of the changes was observed in the prostate glands of the control animals.

COMMENT

Our studies have up to this time revealed no definite reason for the relatively high incidence of urinary calculi in treated animals as compared to the relatively low incidence in the untreated animals. There is evidence, however, that both metabolic and inflammatory factors may be at work.

The chemical analyses of the concretions showed them to be composed of calcium and magnesium phosphates and it is not illogical to postulate that their formation may be related to changes in the metabolism of these substances.

Although the effects of estrogen on the level of calcium in the blood serum are not agreed upon by all workers in this field, definite elevations of these levels have been reported in various birds (19–22) following the injection of large doses over variable periods of time. In mice the available data indicates that the level of serum calcium is only slightly elevated by estrogen injections (26), but there is evidence that the calcium metabolism is altered as a result of such treatment (23, 24, 25).

The relatively high incidence of calculi in animals which received 800, 1200, 1600 and 2000 R.U. of estrogen, as compared to the relatively low incidence in the animals of the other groups, including those which received continuous injections until death, may also be related to changes in the calcium metabolism. It has been shown that the administration of estrogen to mice in large doses produces partial or complete osseous replacement of the marrow cavities of some bones, especially the femora (23, 24). Such bones contain higher concentrations of inorganic substances than the bones of untreated animals, and the ratio of calcium to phosphorus is higher in the treated than in the untreated animals (25). The anatomic changes in the bones have been shown to be at least partially reversible, that is, the bones tend to return to normal following the cessation of treatment (23).

In the experiments reported in this paper the highest incidence of calculi occurred in the animals which received 800 to 2000 R.U. of estrogen and then lived from an average of 8.9 to an average of 4.8 months after the termination of treatment. It is possible, therefore, in these animals that the formation of the calculi was related to the removal of the calcium from the bones during the period after which treatment was terminated.

Preliminary studies would seem to confirm this hypothesis. Chemical analysis of 20 femora showed a significantly larger proportion of ash in the bones of the mice which received continuous injections of estrogen over long periods of time than in the bones of untreated control animals.

A second factor which may also promote the formation of calculi is probably related *a*) to the stasis of urine, *b*) to the metaplasia and desquamation of cells from the mucous membranes of the bladder, prostate, and coagulating glands and *c*) to the infection noted in the genito-urinary tract of most treated males. This belief is supported by the fact that in some treated animals with no gross evidence of calculi, microscopic calcium depositions were present on the surface of necrotic debris and fibrin (fig. 9). These findings would seem to indicate that epithelial hyperplasia with desquamation of cells and urinary tract infection are important factors in the initiation of calculous formation.

This factor may also account for the relatively low incidence of calculous forma-

tion in treated female (3.4%) as compared to treated male mice (43.7%). In the females there are few accessory sex organs closely attached to the urinary tract. Desquamation of epithelial cells into the urinary tract and inflammatory processes initiated by changes in the accessory sex organs are less likely, therefore, to play a part in the formation of calculi in the females than in the males.

SUMMARY

Urinary calculi were found in 4.1% of male, and 5.4% of female control strain C_3H mice.

Urinary calculi were found in 43.7% of male, and 3.4% of female strain C_3H mice treated with varying doses of α-estradiol benzoate in a solution of sesame oil.

The highest incidences (43.3 to 60.0%) of calculous formation occurred in males which had been given from 8 to 20 weekly injections of 100 rat units (0.0165 mg.) of estrogen, after which the animals lived, untreated, for an average of from 8.9 to 4.8 months.

In both treated and control animals the calculi were composed of calcium and magnesium phosphates mixed with organic material.

Pathologic changes, similar to those reported by other investigators, were observed in the genito-urinary tract.

Both metabolic and inflammatory factors may be responsible for the high incidence of calculi in the estrogen treated animals. The available evidence indicated that the metabolic factor was associated with a derangement of calcium metabolism. The inflammatory factor seemed to be related to hyperplasia and desquamation of the genito-urinary tract epithelium and to infection and inflammatory reactions in the urinary tract.

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NOTES AND COMMENTS

EMOTIONAL GLYCOSURIA IN MEDICAL AND DENTAL STUDENTS

Attention was first directed to the possibility that temporary glycosuria may occur in normal individuals as a result of emotional excitement by Cannon et al.[1] who found sugar consistently in the urine of experimental cats exhibiting fear or rage. As it was known that injection of adrenalin gave rise to glycosuria, the physiological glycosuria was explained as a result of increased secretion of adrenalin occurring during strong emotion. Investigating this phenomenon in the human subject Folin[2] examined 34 second year medical students before and after an examination and found one had sugar both before and after the examination. Cannon and Fiske[3] examined the urine of 25 football players after a football game and found sugar in 12, or 48 per cent, five of the positive cases being in substitutes who did not take part in the game.

Edwards, Richards and Dill[4] investigated hyperglycemia during physical exertion and concluded that "Hyperglycemia is uncommon in exercise with little or no emotional stress but common in exercise with emotional stress on the football field." Recently emotional

Date	Class	Number Samples	Number Positive	Per Cent Positive	Number Trace	Per Cent Trace
Dec. 1938	Medical	23	0	0	0	0
April 1939	Dental	40	0	0	3	7.5
Dec. 1939	Medical	29	0	0	8	29.6
April 1940	Dental	43	2	4.7	1	2.3
Dec. 1940	Medical	60	0	0	3	5.0
March 1940	Dental	49	2	4.1	4	8.2
Total		244	4	1.6	19	7.8

glycosuria in Chinese students was studied by Adolph.[5] They determined the blood and urine sugar of 76 male and female students before and after an examination and on a control day. In their study, no emotional hyperglycemia or glycosuria was found.

The clinical significance of temporary emotional glycosuria is emphasized by Johnson[6] who reports 4 cases, 3 in preoperative patients and 1 in an applicant who was undergoing an insurance examination. Watson[7] found that in over 50,000 routine urine examinations in a student health service glycosuria occurred in 1.14 per cent. Further study by the glucose tolerance test of the positive cases revealed an incidence of disturbed carbohydrate metabolism in only 0.17 per cent in the college-age group.

During the past three years, second year medical students and first year dental students were examined for glycosuria following their final examination in the course in Physiology.

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These examinations were fairly difficult and 3 hours in duration. A total of 244 samples were examined by the Benedict qualitative test for sugar. The results were graded in three ways: *negative*, where no color change occurred; *trace*, where a green-blue color occurred; and as *positive*, where a yellow to brown-red color with definite precipitation occurred (1 + to 4 +). Of 244 samples, 4 or 1.6 per cent were definitely positive while 19 or 7.8 per cent showed a trace reaction. Those students who showed a definitely positive reaction were all retested at a later date and found to be free of glycosuria. Judging by these four positive cases the mental stress and anxiety apparently responsible for the glycosuria were also associated with poor results on the examination. Their grades ranged from 33 to 64 as compared to 33 to 93 and 49 to 90 for all students tested in the dental and medical groups respectively.

From these studies on medical and dental students it appears that considerable sugar in the urine, associated with the emotional excitement of written examinations, is not common, occurring in less than 2 per cent of our cases.

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